Scientific Reports

https://doi.org/10.1038/s41598-026-39759-y

Article in Press

Compound heterozygous CHAT gene mutations, a missense and a splice site variant, in two siblings with congenital myasthenic syndrome

Received: 3 June 2025

Accepted: 6 February 2026

Published online: 16 February 2026

Cite this article as: Kikuchi S., Wada N., Mariya T. *et al.* Compound heterozygous CHAT gene mutations, a missense and a splice site variant, in two siblings with congenital myasthenic syndrome. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-39759-y

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Title page

Submitted to Scientific Reports

Manuscript category: Original article

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Abstract

Mutations in the *choline acetyltransferase* (*CHAT*) gene cause congenital myasthenic syndrome (CMS). Episodic apnea is frequently observed in patients with CMS due to *CHAT* mutations (CMS-CHAT), and muscle hypotonia at birth or in early infancy is also common. We report two siblings with compound heterozygous mutations in the *CHAT* gene: c.1231G>A (missense) and c.752+2T>C (splice site). To confirm the splice site mutation induces a splicing variant, we performed a minigene assay and demonstrated that the splice site mutation, c.752+2T>C, results in complete exon skipping. AlphaFold2 analysis predicted that the skipped exon constitutes an α helix, a highly conserved core structural element of ChAT. These structural alterations in ChAT may underlie the clinical phenotype associated with these mutations.

Keywords: congenital myasthenic syndrome (CMS); choline acetyltransferase (ChAT, *CHAT*); splice-site mutation; minigene assay

Introduction

Congenital myasthenic syndrome (CMS) comprises a group of genetically inherited disorders caused by defects at the neuromuscular junction[1, 2]. According to Ohno and coworkers, depending on the mutation site, CMS is classified as presynaptic, synaptic, or postsynaptic[3]. 35 genes have been categorized into 14 groups based on CMS pathomechanisms, clinical features, and treatment responses. Mutations in the *choline acetyltransferase* (*CHAT*) gene fall within the group associated with defective resynthesis of acetylcholine from recycled choline and acetyl-CoA[2]. The most frequently observed presynaptic alterations involve *CHAT* mutations located on chromosome 10q11.2. ChAT catalyzes the synthesis of acetylcholine from acetyl-coenzyme A and choline. Ohno et al. first reported that *CHAT* mutations cause a form of CMS (CMS-CHAT) characterized by life-threatening episodes of apnea[4]. We report two siblings with compound heterozygous *CHAT* mutations, including a missense mutation and a splice-site mutation. The splice-site mutation has not been previously described. Exon skipping of *CHAT* was confirmed by a minigene assay.

Case and Results

Case

Pedigree of the Family

The proband is a 24-year-old male patient, the first child of healthy, non-consanguineous parents. He was born at term without complications but exhibited mildly delayed motor development, achieving head control at 5 months and independent walking at 1 year and 6 months. At 1 year and 8 months, he experienced an episode of apnea with loss of consciousness following a febrile illness, requiring assisted

ventilation. A similar episode requiring ventilation occurred at age 2. Since age 3, he has experienced frequent episodes of generalized muscle weakness and eyelid ptosis triggered by febrile illness, upper respiratory infections, or exertion. Neurological examination was normal except for mild proximal muscle weakness. Cognitive delay was noted (IQ 67). Standard 3Hz repetitive nerve stimulation of the ulnar nerve for 20 s revealed no abnormalities. We did not perform 5-minute stimulation at 10 Hz. Repetitive nerve stimulation and brain MRI findings were normal. No anti-acetylcholine receptor or anti-muscle-specific kinase antibodies were detected. Treatment with pyridostigmine led to improvement in ptosis and muscle strength.

The proband's younger sister is a 14-year-old female, the second child of the same healthy, non-consanguineous parents. Her neonatal period was uneventful. She also had delayed motor milestones, achieving head control at 5 months and walking without support at 1 year and 7 months. At 1 year and 8 months, she experienced an episode of apnea, loss of consciousness associated with fever, and a generalized tonic seizure, diagnosed as a febrile convulsion. At age 2, she had another episode of apnea with loss of consciousness after a febrile illness, requiring assisted ventilation. Since then, she has frequently shown eyelid ptosis and general muscle weakness after febrile illnesses, upper respiratory infections, or physical activity. The neurological exam was normal except for mild proximal muscle weakness at rest. Antibodies against the acetylcholine receptor and muscle-specific kinase were negative. A Tensilon test was conducted, which showed moderate improvement in bilateral ptosis and paroxysmal muscle weakness, indicating a positive response. Pyridostigmine treatment led to improvement in eyelid ptosis and overall muscle strength. Cognitive delay was also noted (IQ 70). Brain MRI findings were normal.

Results

1) Sequence analysis

Six isoforms of *CHAT* mRNA were identified: H, M, N1, N2, R, and S[5]. Nucleotide numbering was based on the first nucleotide of the initial methionine codon in human *CHAT* transcript variant M (NM_020549.5). The two siblings carried compound heterozygous *CHAT* mutations: c.1231G>A (G411R) and c.752+2T>C. The father carried the c.752+2T>C mutation on one allele. The mother carried the c.1231G>A mutation on one allele (Fig. 1A). Glycine at position 411 of ChAT is highly conserved across species, including pig (NP_001001541), mouse (NP_034021), and rat (NP_002264064) (Fig. 1B). The c.1231G>A (G411R) mutation was predicted to be pathogenic by PolyPhen-2.

2) Minigene assay

We could not obtain *CHAT* total RNA; therefore, a minigene assay was performed to compare c.752+2T (wild type) and c.752+2T>C (mutant) constructs. RT-PCR products showed an approximately 250-bp band for the wild-type construct and a lower-molecular-weight band for the c.752+2T>C mutant (Fig. 2A). Sequencing of the wild-type cDNA confirmed correct splicing between exons 4 and 5 and between exons 5 and 6 (Fig. 2B). In contrast, the c.752+2T>C mutant showed exon 5 skipping (Fig. 2C). This skipping did not result in a frameshift or premature stop codon. The region spanning amino acids 233 to 250 in ChAT, which is affected by exon 5 skipping, contains 16 of 18 residues that are highly conserved among pig, mouse, and rat (Fig. 2D).

3) Protein structure prediction by AlphaFold2

AlphaFold2-based structural prediction showed that exon 5, corresponding to residues Arg233 to Asn250 in ChAT, forms an α-helix in the wild-type protein. This α-helix is absent in the ChAT protein lacking exon 5 due to the c.752+2T>C splice site mutation (Fig. 2E).

4) Verification of disease-causing potential by in silico analysis

We did not obtain full-length *CHAT* RNA; our data had limitations to explain that the compound heterozygote mutation in *CHAT* induced CMS. Therefore, we used in silico analysis to verify the pathogenic potential of the identified mutations in ChAT. We used Mutation Taster 2025[5], SIFT[6], polyphen-2[7, 8], and Splice AI[9] (Table 1). The results showed that the mutation p.G411R was deleterious and probably damaging in Mutation Taster 2025, SIFT, and PolyPhen-2, respectively. The exon 5 skipping was deleterious and damaging in Mutation Taster 2025 and SIFT. We did not use PolyPhen-2 for exon 5 skipping because it handles point mutations, not large deletions. The results suggested that these mutations might have abnormal functions. Finally, we used Splice AI for the c.752+2T>C splice site mutation to confirm donor loss score, and the score was 0.73.

Discussion

CMS is a heterogeneous group of genetic disorders that affect the neuromuscular junction at presynaptic, synaptic, or postsynaptic sites. *CHAT* gene mutations fall under the presynaptic category. In the seminal report by Ohno et al., *CHAT* mutations were initially identified as the cause of CMS presenting with episodic apnea[4]. Episodic apnea and respiratory distress are hallmark features of CMS-CHAT and typically manifest as muscle hypotonia at birth or in early infancy. These symptoms are often triggered by fever, infection, or stress. In both cases presented here, the siblings experienced

recurrent apnea and generalized muscle weakness precipitated by febrile episodes since infancy. Both cases yielded a positive Tensilon test. Based on clinical presentation and pharmacologic response, CMS-CHAT was suspected, and genetic testing confirmed compound heterozygous *CHAT* mutations.

To date, over 50 CMS-CHAT cases have been reported[10], involving 42 distinct mutations, including missense, nonsense, frameshift, deletion, and duplication variants[11]. Clinical severity varies substantially, even among family members carrying the same genotypes[12]. Arredondo et al. [13] reported that mutations that reduce enzyme expression and cause severe kinetic impairment are associated with more severe clinical phenotypes. Murtazina et al.[12] further demonstrated that identical *CHAT* genotypes can lead to a broad spectrum of clinical severity.

Intellectual disability has also been reported in several CMS-CHAT cases. Schara et al.[14] observed that all neonatal-onset cases and three of seven infantile-onset cases exhibited cognitive impairment. Interestingly, the most severe case in Schara's report carried a homozygous p.G417R *CHAT* mutation, which is located near our mutation p.G411R. Two reports showed brain images and concluded that reduced brain volume likely resulted from hypoxic injury associated with episodic apnea[12, 14]. In contrast, our cases showed intellectual disability despite normal brain MRI findings. These findings suggest that intellectual disability is not solely attributable to hypoxic brain injury but may also reflect the direct consequence of ChAT deficiency on the central nervous system, where the enzyme is widely expressed. The two cases described here demonstrated moderate intellectual disability despite normal brain MRI findings and required assisted ventilation after 1 year of age, indicating that hypoxia alone is unlikely to be the primary contributor.

The siblings presented here carried compound heterozygous *CHAT* mutations: a missense mutation (c.1231G>A, p.G411R) and a novel splice site mutation (c.752+2T>C). The p.G411R mutation has been previously reported in a female patient exhibiting a milder phenotype characterized by distal weakness beginning at four years of age[15]. Notably, p.G411R is located at p.R420, a residue at which the p.R420C variant was shown by Ohno et al. to destabilize protein folding despite being distant from the active and binding site, p.H442[4]. Although it is difficult to conclude that the mutation, p.G411R, induces CMS directly, according to the previous report[15] and the placement of p.G411 in ChAT, p.G411R is presumed to have a negative effect on ChAT function. The splice site mutation has not been reported previously. Because ChAT expression is restricted to human mononuclear leukocytes and leukemic T-cell lines in peripheral blood[16], endogenous mRNA isolation from whole blood cells was not feasible. Therefore, we employed a minigene assay to assess whether the c.752+2T>C mutation caused aberrant splicing. The assay revealed exon 5 skipping without inducing a frameshift or premature termination codon. Thus, the resulting transcript is likely to escape nonsense-mediated mRNA decay. We understand the importance of experiments for kinematic analysis in ChAT mutations; however, we demonstrated that the minigene assay was very useful for confirming that a splice-site mutation induces a splice variant when the available sample expresses insufficient target mRNA.

AlphaFold2-based structural modeling revealed that exon 5 encodes an α-helix spanning residues 233–250. This helix was absent in the exon 5-deleted protein. Cai and colleagues[17] provided a detailed three-dimensional (3D) structure of rat ChAT, which corresponded well to that of human ChAT. The deleted α-helix induced by the exon 5 skipping corresponds to the sixth α-helix (α6) in rat ChAT. Rat ChAT has two structural

domains: residues 102-401 make up the N domain, and residues 18-101 and 402-617 form the C domain. Both domains have α- and β-folds and share a common core structure. The α6 contributes one of the three helices that stabilize the common core structure of the N domain, together with α8 and α13, which correspond to α16, α18, and α22 in the C domain[12]. The critical importance of this region is underscored by previous reports: Shen et al.[18] described a missense mutation (A255T) that reduced the ChAT turnover number, and Liu et al.[12] reported a patient with a large deletion spanning exons 4–6 who died at 10 months from respiratory failure. Taken together, these findings support the essential role of exon 5 in maintaining ChAT structure integrity. We propose that the deletion of exon 5, in conjunction with the p.G411R mutation on the opposite allele, plays a significant role in the etiology of CMS-CHAT in these cases.

Our experimental results did not provide direct evidence that the combined heterozygous mutations, p.G411R and exon 5 skipping, would induce ChAT dysfunction kinetically. In addition, we were unable to obtain full-length *CHAT* cDNA, so we could not proceed with additional experiments to explore ChAT protein instability and kinetic dysfunction. Instead of analyzing the ChAT protein function or stability, we used some in silico analysis. The results showed that both p.G411R and exon 5 skipping mutations predictably cause damaging effects, suggesting that compound-damaging mutations might induce abnormal ChAT function.

In conclusion, the identified ChAT mutations, p.G411R and exon 5 skipping, may contribute to CMS in the siblings described here. To the best of our knowledge, exon 5 skipping detected by the minigene assay represents the first reported splice-site mutation in CMS-CHAT.

Methods

Ethics

All methods were carried out in accordance with relevant guidelines and regulations. Written informed consent for the study, including gene analysis, was obtained from the patients' parents, and the patients' case information and blood samples were collected in accordance with the guidelines of Tomakomai City Hospital. The genome study was conducted according to the Ethical Guidelines for Medical and Biological Research Involving Human Subjects in Japan. The mini-gene assay was approved by the safety committee for recombinant DNA experiments of Sapporo Medical University (No. R7-101).

DNA extraction, whole-exome sequencing, PCR, and Sanger sequencing

Blood samples were collected for DNA extraction after obtaining written informed consent from the two siblings and their parents. For whole-exome sequencing (WES), exonic regions were enriched using the MGIEasy Exome Capture V5 Probe Set and sequenced on an MGI DNBSEQ-T7 platform with 150-bp paired-end reads, achieving approximately 100× mean coverage across the targeted exome. Alignment and variant calling were conducted utilizing BWA-MEM and the Genome Analysis Toolkit (GATK v3.5), in accordance with the GATK Best Practices guidelines, using the human reference genome hs37d5. Only rare variants with a minor allele frequency (MAF) < 0.01 were included. Variant filtration was conducted using an in-house database. For Sanger sequencing, the target regions were amplified by polymerase chain reaction (PCR) using KOD DNA polymerase (TOYOBO Co., Ltd., Tokyo, Japan) and primer sets listed in Table 2. Sequencing was performed using the standard Sanger method.

Minigene assay

To construct the *CHAT* minigene, a 5-kb genomic fragment spanning from the 5' end of exon 4 to exon 6 was amplified by PCR using the primer sets listed in Table 2. The forward primer included a Kozak sequence and a start codon, while the reverse primer contained a stop codon. The PCR product was cloned into the pcDNA3.1 expression vector (pcDNA3.1-h*CHAT*; Thermo Fisher Scientific Inc., Waltham, MA, USA) (Supplementary Fig. S1). Sanger sequencing confirmed whether the pcDNA3.1-h*CHAT* constructs contained the wild-type (c.975+2T) or mutant (c.975+2C, splice site mutation) sequence. Both wild-type and mutant constructs were transfected into cultured HEK293 cells (Thermo Fisher Scientific Inc., Waltham, MA, USA). After 2–3 days, total RNA was extracted and reverse-transcribed into complementary DNA (cDNA). The cDNA was amplified by PCR, and the PCR products were resolved on a 1% agarose gel. Purified PCR products were sequenced using the Sanger method.

Protein structure prediction by AlphaFold2

We used LocalColabFold, an offline implementation of AlphaFold2, to model the structural consequences of the c.752+2T>C mutation on the protein. Structural models were visualized using PyMOL (The PyMOL Molecular Graphics System, Version 2.5; Schrödinger, LLC)[19, 20]. Local conformational differences between the wild-type and mutant ChAT proteins were evaluated, focusing on α-helices and β-sheets surrounding the mutation site.

Prediction of pathogenicity

We used Mutation Taster 2025 (https://www.mutationtaster.org/), SIFT (https://sift.bii.a-star.edu.sg/), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) to predict whether the mutations were damaging or not. In addition, we checked the ratio of donor loss in c.752+2T>C mutation using Splice AI

(https://spliceailookup.broadinstitute.org/). In Mutation Taster 2025, we used two analyses: chromosomal position (GRCH38) and specific transcript. Regarding the mutation, c.1231G>A, we entered Chr10:49646624G>A or c.1231G>A into chromosomal position and specific transcript, respectively. For c.752+2T>C, we entered Chr10:49622152T>C or c.699_752del for the chromosomal position and specific transcript, respectively, because we have already confirmed skipping exon 5 (c.699_752del). In SIFT, we used GRCh38 and entered each mutation position. PolyPhen-2 was used for analysis in only c.1231G>A, because PolyPhen-2 supported only point mutation.

Declaration

Author contribution

S.K. and N.T. designed the study and wrote the manuscript with support from M.K. and Y.O., H.K., S.F., Y.N., and T.O. performed the genetic analysis and evaluation. T.M. and M.K. conducted the three-dimensional structure building and analysis. S.K. and N.W. carried out the minigene assay. N.T. and M.K. collected the clinical data. All authors approved the final manuscript.

Data Availability

All data generated or analysed during this study are included in this published article and its Supplementary Information Files. The original data from this study are available from the corresponding author upon reasonable request. The accession numbers of c.752+2T>C and c.1231G>A are SCV006087544 and SCV006087545 on ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), respectively.

Funding

This work was supported by JSPS KAKENHI Grant Numbers 22K11315 and 25K14457 (to S.K.), 25K21078 (to N.W.), and 24K02208 (to Y.O.), and by AMED under Grant Number JP24ek0109760.

Acknowledgements

We would like to thank Prof. Kinji Ohno (Nagoya University of Arts and Sciences) for valuable discussions. We also thank Dr. R. Ichikawa and Dr. H. Hino for his advice and K. Iwamura and Y. Moriya for their support. We would like to thank Editage (www.editage.jp) for English language editing.

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Figure and Table legends

Fig. 1

A. Pedigree of the family showing the compound heterozygous mutations (c.1231G>A and c.752+2T>C) in the two affected siblings. The father carries the c.752+2T>C mutation, and the mother carries the c.1231G>A mutation.

B. Multiple sequence alignment of amino acid residues 401–421 of ChAT from human, pig, mouse, and rat. Gray highlights indicate residues identical to human ChAT. The red rectangle marks glycine at position 411, which is mutated to arginine (G411R) in the affected individuals.

Fig. 2

A. Left panel is whole gel and gray rectangle is shown at higher magnification in right panel. Agarose gel electrophoresis showing a 250-bp band for the wild-type construct and a lower molecular weight band for the c.752+2T>C mutant.

B, **C**. Sanger sequencing results of the minigene assay for the wild-type (**B**) and c.752+2T>C mutant (**C**) alleles. Red-labeled residues in **B** correspond to amino acids skipped in **C**.

D. Multiple sequence alignment of amino acid residues 220–260 of ChAT from human and single isoforms identified in pig, mouse, and rat. Gray highlights indicate residues identical to human ChAT. The red rectangle marks the region affected by exon 5 skipping.

E. AlphaFold2-based 3D structural models of wild-type ChAT (cyan) are shown in the upper left, middle, and right cartoons, with residues R233 to N250 highlighted in yellow. Corresponding models of the c.752+2T>C mutant protein lacking this region are shown

in the lower left, middle, and right cartoons (magenta). An overlay of wild-type and mutant structures is displayed in the middle cartoon. The right cartoon ("Pick up") shows a magnified view of residues R233 to N250 in the wild-type structure to illustrate the structural loss caused by exon 5 skipping.

Table 1

Result of *in silico* analysis

Mutation	752+2T>C	1231G>A
Mutation Taster 2025	Deleterious	Deleterious
SIFT	Damaging	Damaging
Polyphen2	-	probably damaging
SpliceAI	Donor Loss, 0.73	-

Table 2

Primer sets for polymerase chain reaction (PCR)

Target region in *CHAT*	Forward / Reverse	Sequence
Exon 5	Forward	5'-tcctctcccttccccaagag-3'
	Reverse	5'-gtgcttgccacaggaaaagg-3'
Exon 9	Forward	5'-ttccactgatgtgggctgac-3'
	Reverse	5'-gctgcaggaaggttagggag-3'
Exon 4-6	Forward	5'-ccaccatggccgtgatctttgctcgg-3'
	Reverse	5'-ttacatgatgctgctgttctgag-3'