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Research article

A novel *MMUT* splicing variant causing mild methylmalonic acidemia phenotype

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ABSTRACT

Objectives: Methylmalonic acidemia (MMA) is a rare inborn genetic disorder that is characterized by increased levels of methylmalonic acid in blood plasma and urine. Isolated methylmalonic acidemia is one of the most common types of MMA and is caused by mutations in the gene encoding methyl-malonyl coenzyme A mutase (*MMUT*). In this study, we investigated the possible mechanisms underlying the symptoms of isolated MMA in a patient by molecular analysis.

Methods: PCR amplification and Sanger sequencing analysis was performed to identify variants in the *MMUT* gene in the proband and his family. Furthermore, minigene constructs were generated to validate the splicing defects in the *MMUT* gene variant identified in the proband.

Results: The 3-year-old patient was admitted to the hospital with symptoms of MMA, including fever, convulsions, and vomiting. He showed metabolic acidosis, high levels of methylmalonic acid in blood and urine, and normal blood homocysteine levels. Genetic analysis demonstrated that the patient was a compound heterozygous carrier of two variants in the *MMUT* gene: a missense c.278G > A variant that has already been reported in a patient with the severe mut^0 phenotype; and a novel splice site variant c.2125-2A > G. RT-PCR analysis showed that, while the novel variant clearly alters splicing, a minor amount of a full-length transcript is generated, suggesting that a wild-type protein may be produced although at a lower quantitative level. The patient's condition improved after treatment with vitamin B12. Serious complications were not reported during follow-up at age 5.

Conclusions: We identified a novel splice site variant that partially disrupts normal splicing of the *MMUT* pre-mRNA. Production of a reduced amount of full-length transcript is responsible for the mild clinical phenotype observed in this patient. Functional studies have proven useful in exploring the genotype-phenotype association and in providing guidance for the genetic diagnosis of MMA.

Abbreviations: MMA, methylmalonic acidemia; MCM, methyl malonyl-CoA mutase; MCEE, methyl malonyl-CoA epimerase; TCA, Tricarboxylic Acid Cycle; HGMD, Human Gene Mutation Database; ACMG, American Society for Molecular Genetics and Grnomics; PVS1, pathogenic very strong 1.

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1. Introduction

Methylmalonic acidemia (MMA) is an autosomal recessive inborn genetic disorder characterized by elevated levels of methylmalonic acid in the blood, urine, and cerebrospinal fluid [1]. The global incidence of MMA ranges from 1:50,000 to 1:200,000 births [2,3]. MMA is caused by the inability to metabolize methylmalonyl coenzyme A because of deficiency in enzymes such as methyl malonyl-CoA mutase (MCM) or methyl malonyl-CoA epimerase (MCEE) [4], or deficiency in proteins that are involved in the synthesis and transport of adenosylcobalamin (cofactor of MCM), including cblA, cblB, cblC, cblF, cblD, and cblX [5]. Patients with MMA are classified into the two types based on the plasma homocysteine levels: (1) isolated methylmalonic aciduria (without hyperhomocysteinaemia) and (2) combined methylmalonic aciduria (with hyperhomocysteinaemia) [6]. Currently, isolated methylmalonic aciduria is associated with mutations in at least four genes. The most common mutations in patients with isolated MMA occur in the *MMUT* gene that encodes the MCM protein (MIM # 251000) [7,8]. MMA is also caused by mutations in the *MMAA* gene that encodes cblA (MIM # 251100), *MMAB* gene that encodes cblB (MIM # 251010), and *MCEE* gene that encodes methyl malonyl-CoA isomerase (MIM # 251120) [9]. Mutations in the *MMAA* and *MMAB* genes disrupt the synthesis and metabolism of 5′-deoxyadenosylcobalamin, an essential cofactor for the functioning of methylmalonyl-coenzyme A [10].

MCM is an adenosylcobalamin-dependent mitochondrial enzyme that plays an important role in the metabolism of isoleucine, threonine, valine, and odd-chain fatty acids. In the mitochondria, MCM catalyzes the isomerization of methyl malonyl-CoA into succinyl-CoA, which subsequently enters the tricarboxylic acid (TCA) cycle [11]. MCM protein deficiency causes abnormal accumulation of methylmalonic acid, propionic acid, and methyl citrate, and damages multiple organs and cells, including the neurons, liver, kidneys, and the bone marrow [12]. Two categories of MMA patients' subtypes have been identified based on the MCM apoenzyme deficiency: (1) mut^0 type with complete loss of MCM protein activity; (2) mut^- type with low residual MCM protein activity [13]. Children with the mut^0 type MMA are associated with $\leq$0.1% MCM enzyme activity, show features of metabolic acidosis in the first week of life, and are at greater risk of early death [14]. Children with mut^- type MMA show disease symptoms within a few months or years of life and are susceptible to recurrent ketoacidosis because of infections with one year after birth [15].

In this report, we describe an isolated methylmalonic aciduria patient with a novel pathogenic splicing *MMUT* variant who displays a mild MMA phenotype. Our data supports the hypothesis that this novel splice site variant involves leaky splicing of the mutant *MMUT* gene.

2. Materials and methods

2.1. Patients and samples

The proband was a 3-year-old Chinese boy who was admitted to the Tianjin Children's hospital. Since he was a minor child, his parents provided written informed consent after interview. This study was approved by the Ethics Committee of the Tianjin Children's

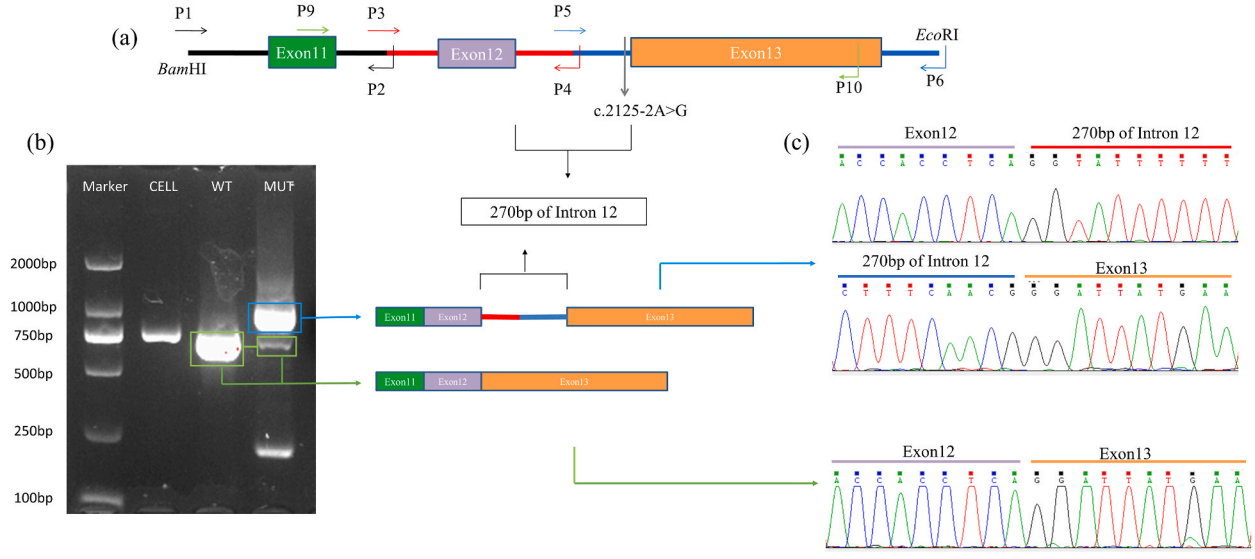

Fig. 1. Aberrant splicing caused by the c.2125-2A > G variant. **(a)** Schematic representation of the 1747 bp oligonucleotide sequence inserted into the expression vector. The exons are denoted by boxes. The black, red, and blue lines indicate introns flanking exons 11, 12, and 13, respectively. The gray arrow indicates c.2125-2A > G. The position of primers P1–P6 and P9–P10 are also shown. **(b)** Agarose gel electrophoresis of the RT-PCR products. Lane 1, D2000 bp marker; lane 2, untransfected HEK293T cells; lane 3, HEK293T cells transfected with the wild-type *MMUT* plasmid vector, and lane 4, HEK293T cells transfected with the variant *MMUT* plasmid vector. **(c)** Sequencing analysis results show insertion of a hybrid 270 bp intronic sequence.

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2.2. Genetic analysis

Fasting venous blood was collected from the patient and his parents. The genomic DNA was extracted from the blood samples using the Blood Genomic DNA Kit (CWBIO Biotech Co., Ltd) and stored at −20 °C. The concentration and quality of the genomic DNA samples was estimated using the NanoDrop 2000 spectrophotometer. We used the following *MMUT* transcripts for our analysis: ENST00000274813.4 and NM_000255.4. The amplification of *MMUT* exons was performed using 13 pairs of primers as described previously [16]. Sanger sequencing was performed by the GENEWIZ Company.

2.3. Vector constructs for the minigene assay

The pcDNA3.1-HisA vector plasmid was used for the minigene assay. This vector contained a CMV promoter, BGH polyadenylation sequence, and multiple cloning sites. An oligonucleotide sequence of 1747 bp (Fig. 1a) was cloned into the *Bam*HI and *Eco*RI sites of the pcDNA3.1-His vector by homologous recombination with the ClonExpress Ultra One Step Cloning Kit (Vazyme Biotech Co., Ltd) according to the manufacturer's protocol. This oligonucleotide sequence containing intron-exon-intron features were amplified. Each exon was flanked by ~150 bp of intron sequences. The amplicon 11 (upstream sequence of 197 bp + Exon 11 sequence of 149 bp + downstream sequence of 126 bp), amplicon 12 (upstream sequence of 118 bp + Exon 12 sequence of 168 bp + downstream sequence of 149 bp), and amplicon 13 (upstream sequence of 122 bp + Exon 13 sequence of 579 bp + downstream sequence of 139 bp) were amplified by PCR. The reference recombined vector was constructed with primers P1–P6 (Fig. 1a). The mutant recombined vector was constructed using the Fast Site-directed Mutagenesis Kit (Tiangen Biotech Co., Ltd) with primers P7–P8. Plasmids were sequenced to confirm the presence of directed mutations.

2.4. Cell culture and transfection

Human Embryonic Kidney 293T (HEK293T) cells were cultured in six-well cell culture plates with DMEM (Gibco, Thermo Fisher) containing 10% fetal bovine serum. The reference and variant plasmid constructs were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher) according to manufacturer's protocol.

2.5. RT-PCR analysis

After 48 h, total RNA was isolated from transfected cells using Trizol (Invitrogen, Thermo Fisher) according to manufacturer's instructions, and amplified by RT-PCR using the minigene PCR primers P9–P10 (Fig. 1a). The PCR products were separated by electrophoresis on a 1.5% agarose gel stained with 1% Gel-red (Biotium). Finally, RT-PCR products were sequenced. All the primers used in the minigene assay are listed in Table 1.

3. Results

3.1. Clinical findings confirm a case of isolate MMA

The patient was a three-year-old boy who was admitted to the Children's hospital because of self-reported fever, vomiting and convulsions. The boy presented with unconsciousness and turning of the eyes during convulsions. The early psychomotor development was normal and there was no previous history of cerebral lesions. After admission, the patient demonstrated symptoms of lassitude, lethargy, and slow response after pain stimulation. The cranial MRA did not show any abnormalities. The electroencephalogram monitoring indicated slow waves in the occipital region. The brain MRI (T2WI/FLAIR) showed patchy hyperintense shadows in the

Table 1
Minigene assay associated primers.

Primers	Explanation	F/R	Sequence 5′-3′
P1	Amplicon11	F	cttggtaccgagctcggatccTAACTATGTAACAAGCCTGCACATTAA
P2		R	agacttcatgCAGTGGCTACATACCAGTTACCAGG
P3	Amplicon12	F	gtagccactgCATGAAGTCTTTGCCCATTAGTATG
P4		R	ctactggcCCACTTTTAGACCTTGTAGAATTTATTTAG
P5	Amplicon 13	F	gtctaaaagtggGCCAGTAGTATACCAGTTGAGAAGGTT
P6		R	tgctggatatctgcagaattcTTGAAGAAATATACTGTCAAATTCCCC
P7	Site-specific mutation	F	TTAAACACATTCTTTCAACGGGATTATGAATT
P8		R	CGTTGAAAGAATGTGTTTAATTAATAAGAGC
P9	Minigene PCR primers	F	GCTGATCTTGGTTTTGATGTGG
P10		R	CTGGTGCTCAAGACAATGTAATGC

Small letters indicate the homologous arm sequence (including cleavage sites of restriction enzyme) and capital letters indicate the intron region of the amplicon.

bilateral parietal white matter. Furthermore, multiple linear long T1 and T2 signal shadows were observed in the splenium of the corpus callosum and the white matter area of the bilateral parietal lobe. Blood gas analysis demonstrated metabolic acidosis (pH 7.27–7.42, BE-18.5–6.7 mmol/L). Blood homocysteine levels were normal at 6.3 μmol/L. Gas Chromatograph/Mass Spectrometer (GC/MS) analysis showed increased level of methylmalonic acid in the urine during an acute episode (MMA ratio: 325.5). The MMA ratio in urine decreased significantly to 91.3 after an intramuscular injection of vitamin B12 (dose of 1 mg/qd). The patient was followed-up at 5 years of age and was asymptomatic with satisfactory growth and development that was comparable with children of the same age.

3.2. *The patient is compound heterozygous carrier of two variants in the MMUT gene*

Sanger sequencing was performed to determine the cause of isolated methylmalonic acidemia. Since the patient showed symptoms of isolated MMA, we prioritized analysis of *MMUT* variants. Sanger sequencing results demonstrated a missense variant c.278G > A (p.R93H, rs121918251) in the exon 2 of the *MMUT* gene in the proband. This variant was heterozygous and was inherited from his father and was associated with the MMA mut^0 type. This variant has never been reported in East Asia and the frequency in the general population is 0.0006364 (gnomAD v2.1.1:https://gnomad.broadinstitute.org/variant/6-49426902-C-T?dataset=gnomad_r2_1). The patient and his mother also showed the novel c.2125-2A > G variant located interestingly within the acceptor splice site of intron 12 in the *MMUT* gene (Fig. 2). Therefore, minigene assay was performed to determine if this mutation affected splicing.

3.3. *The c.2125-2A > G variant in MMUT alters significantly but not completely the biosynthesis of a full-length transcript*

Sequencing analysis was performed for all the RT-PCR products that were generated in the minigene assay. Gel electrophoresis (Fig. 1b) of the RT-PCR products showed a single band for the wild-type with an expected size of 745 bp, while the variant showed a similar band with a much lower intensity accompanied by a major larger product. Sanger sequencing revealed that this similar band was consistent with the wild-type after sequence alignment and this analysis results suggested that a full-length transcript may be produced in the variant condition. Moreover, sequencing results showed the other major larger product for the mutant was a hybrid

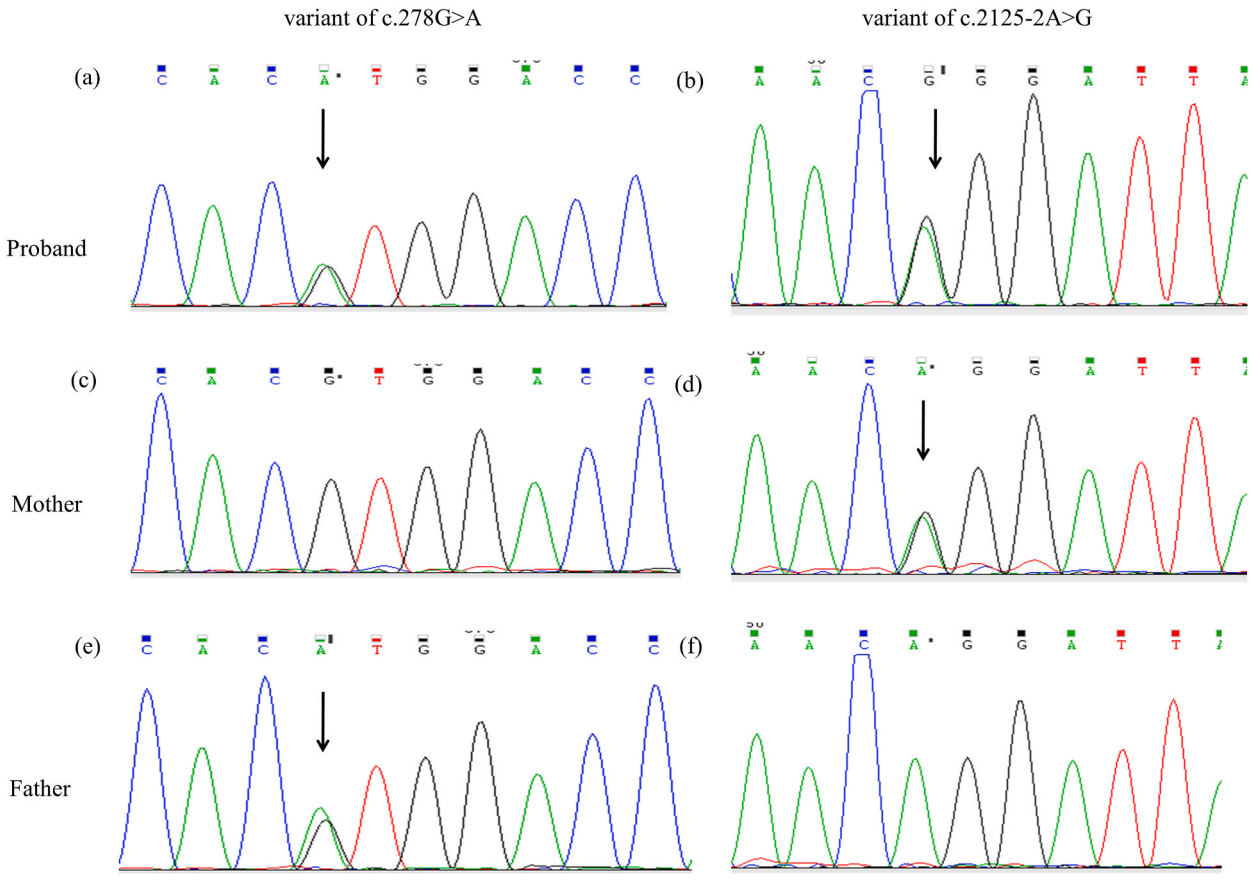

Fig. 2. Sanger sequencing of the proband *MMUT* gene show compound heterozygous variants (a and b). The c.2125-2A > G variant is inherited from the mother (c and d), the c.278G > A variant is from the father (e and f). Black arrows denote variant positions.

intron of 270 bp retained in the coding region between exons 12 and 13 (Fig. 1c), which was considered deleterious compared to the normal splicing pattern of wild-type. Incidentally, the other minimal product in lane "MUT" was a non-specific amplification, whereas the band observed in lane "CELL" was *MMUT* gene-specific endogenous product.

Based on the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines published in 2015 [16], this result meets ACMG criteria for PVS1 (very strongly pathogenic) combined with further applicable evidence for PM3 (moderately pathogenic) and PP4 (supporting pathogenic). Therefore, we conclude that this *MMUT* variant is pathogenic.

4. Discussion

According to the Human Gene Mutation Database (HGMD), 443 different variant types have been identified for the human *MMUT* gene, including 280 missense/nonsense variants, 34 splicing variants, 107 small deletions/insertions, and others (https://portal.biobase-international.com/hgmd/pro/gene.php?gene=MMUT; OCT 09, 2020). More than 50% of the *MMUT* variants cause mut^0 type of MMA and these mutations are mostly found in exons 2, 3, 6, 11 and 12, especially exons 11 and 12, which encodes the cobalamin binding domain sequence in the MCM protein [17]. In the patient described here, we identified a *MMUT* variant c.278G > A based on the molecular genetics analysis in the exon 2 of *MMUT* and was previously reported to be associated with a severe phenotype [18]. We also identified a novel variant c.2125-2A > G. We used the minigene assay to characterize the effect of the c.2125-2A > G variant and observed that this alteration maintains the production of a full-length transcript, although at a much lower level than in wild-type conditions. The maintenance of a full-length transcript is thought to result in the biosynthesis of a reduced amount of normal protein. Our data demonstrated that the proband carried two unequivocally pathogenic variations in the *MMUT* gene, which contributed to the manifestation of isolated MMA.

The clinical symptoms of MMA include lethargy, ketoacidosis, and hypoglycemia or hyperglycemia, and tend to recur; *MMUT* gene variants usually cause relatively severe MMA symptoms [19]. However, onset at 3 years of age, age-appropriate mental and psychomotor development, and mild symptoms in this patient were not in accordance with our initial expectations. The mild symptoms observed in this patient appear to correlate well with the experimental analysis showing that the *MMUT* allele carrying the c.2125-2A > G variant has a partial deleterious defect on splicing.

Patients with *MMUT* variants show comparatively unfavorable prognosis in comparison with the other MMA subtypes. Therefore, these patients require effective therapeutic interventions. It is evident that cobalamin, a coenzyme of MCM, enhances the enzymatic activity of MCM [13]. Hence, cobalamin is a viable therapeutic option for the mut-negative (mut^-) type of MMA. The efficacy of vitamin B12 treatment is highly variant and is dependent on the type of *MMUT* gene variant in the patients [20]. Our patient showed a less severe phenotype and demonstrated good response when supplemented with hydroxocobalamin. This suggested that the biosynthesis of the wild-type *MMUT* mRNA for the normal protein and the MCM enzymatic activity was not lost completely. This genotype–phenotype association further enhances our understanding of methylmalonic acidemia and provides guidance for selecting an appropriate treatment strategy and performing accurate prognostic assessments.

There are some limitations to this study. The current minigene design forces the splicing machine to use a cryptic splice acceptor site in the intron 12, resulted in intron retention that is artificial and not representative of the real situation in vivo. In addition, we did not perform additional studies on protein levels. But in general, minigene assay is very useful for assessing the impact of variants, especially if sufficient patient RNA is not available.

In conclusion, the pathogenic c.2125-2A > G splice site variant of the *MMUT* gene is leaky and results in low expression levels of *MMUT* transcripts, which helps explain the mild clinical phenotype. Our results confirm the significance of functional studies of variants that may affect splicing. The identification of *MMUT* variants can guide the genetic diagnosis of MMA and provide a theoretical basis for further treatment, follow-up studies and clinical strategies.

Ethical Approval and consent to participate

The study protocol was approved by the Ethics Committee of Tianjin Children's Hospital (the Ethics Committee of Tianjin Children's Hospital; 2022-LXKY-009). Written informed consent to participate was obtained from the patient's parents.

Consent for publication

Written informed consent was obtained from the patient's parents for publication.

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Data availability statement

All data relevant to the study are included in the article.

CRediT authorship contribution statement

Xinjie Zhang: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Xiaowei Xu:** Writing – original draft, Methodology, Investigation, Data curation. **Jianbo Shu:** Writing – review & editing, Formal analysis. **Xiufang Zhi:** Software, Methodology. **Hong Wang:** Resources. **Yan Dong:** Investigation. **Wenchao Sheng:** Software. **Dong Li:** Funding acquisition, Conceptualization. **Yingtao Meng:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Chunquan Cai:** Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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