A rare mutation c.1663G>A (p.A555T) in the MMUT gene associated with mild clinical and biochemical phenotypes of methylmalonic acidemia in 30 Chinese patients

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Research

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Abstract

Background Methylmalonic acidemia is an inherited organic acid metabolic disease. it involves multiple physiological systems and has variable manifestations. The primary causative gene *MMUT* carries wide range of mutations, and one of them, c.1663G > A (p.A555T), is considered to be of an extremely rare type. So far, little is known about the clinical features of patients carrying this mutation. In the present study, we aimed to define the clinical and biochemical features of the patients with this genotype.

Methods Among 328 mut type methylmalonic acidemia patients from multiple hospitals in China, we collected 30 patients sharing the mutation c.1663G > A (p.A555T) in the *MMUT* gene. Their clinical characteristics and biochemical index were described in detail and compared with methylmalonic acidemia patients without this variant.

Results Most of these patients were diagnosed via newborn screening (26/30), treated in a timely manner, and kept healthy (24/30). Disease onset occurred in 7 patients. Mental retardation occurred in 4 patients. Vitamin B12 is responsive in 100% of these patients (29/29). The blood propionylcarnitine, blood propionylcarnitine/acetylcarnitine ratio, urinary methylmalonic acid, urinary methylcitric acid before and after treatment in c.1663G > A (p.A555T) carrying patients were much lower than those in non-c.1663G > A (p.A555T) carrying patients.

Conclusion Compared to patients with other mutations in the *MMUT* gene, patients with the c.1663G > A (p.A555T) mutation showed later onset, milder clinical phenotype, lighter biochemical abnormalities, better vitamin B12 responsiveness, lower morbidity, easier metabolic control, and thereby better prognosis. Newborn screening project plays an important role in early diagnosis, treatment, and prognosis of these patients.

Introduction

Methylmalonic acidemia (MMA) is a series of inherited organic acid metabolic disorders. The primary defect occurs in methymalonyl-CoA mutase (MCM) or its cofactor, adenosylcobalamin [1], with the main genetic mode autosomal recessive inheritance. The accumulation of methylmalonic acid and abnormal metabolites causes various clinical symptoms [2]. The incidence of MMA ranges from 1:48,000 to 1:250,000 worldwide [3, 4]. The incidence of MMA in China varies significantly from region to region and was reported to be 1:38,667 in Shanghai [5], 1:46,531 in Zhejiang province [6], 1:6,032 in Henan province [7], 1:40166 in Jiangsu Suzhou district [8], 1:16,883 in Jiangsu Xuzhou district [9] and 1:5589 in Shandong Jining district [10]. The expanded screening program for newborns by tandem mass spectrometry (MS/MS) is currently performed in an increasing number of regions of China. The advancing use of MS/MS in newborn screening and identification of clinically suspected cases beneficially serve for proper and timely diagnosis of MMA.

According to the biochemical manifestation, MMA can be classified into two common types: isolated MMA and MMA combined with hyperhomocysteinemia. Isolated MMA accounts for generally 30% of the total MMA in China [6]. The majority of patients with isolated MMA present clinical symptoms and biochemical abnormalities, such as poor feeding, vomiting, poor weight gain, and convulsion within the first few days or months of life. Life-threatening acute metabolic decompensation may occur intermittently, often precipitated by catabolic factors such as infection and stress. The overall prognosis is generally poor, with neurologic and renal impairment [11]. Most cases of isolated MMA are caused by the mutation in the *MMUT* gene, which encodes the protein MCM, and few incidences are due to the changes in other genes, such as MMAA,MMAB, and others [12]. The *MMUT* gene carries a wide variety of mutations. The mutations spectrum differs significantly in diverse races. For example, c.349G > T (p.E117X), c.385 + 5G > A (IVS2 + 5G > A), c.1106G > A(p.R369H), c.1481T > A (p.L494X), and c.2179C > T (p.R727X) are five relatively frequent mutations in Japan [13]. Indians have many kinds of mutations and c.1863A > T (p.K621N), c.1943G > A (p.G648D), and c.1889G > A(p.G630E) are relatively frequent [14]; c.322C > T (p.R108C) was identified to be frequent in Hispanic patients, while c.2150G > T (p.G717V) was identified as frequent in black patients [15]. In China, the most common mutations include c.729_730insTT(p.D244Lfs*39), c.1106G > A (p.R369H), c.323G > A (p.R108H), and c.1107dupT (p.T370Yfs*22) [9, 16]. The c.1663G > A (p.A555T) mutation in the *MMUT* gene is rare, which is so far reported only twice in two patients[17, 18]. Limited information is available on the clinical and biochemical characteristics of patients carrying this mutation. In the present study, we examined 30 isolated MMA patients carrying this mutation from multiple hospitals during the last 15 years. We performed a retrospective chart review of their clinical data in detail, including molecular diagnosis, metabolites, treatment, and outcomes, and compared them with those of patients carrying other mutations in the *MMUT* gene, in order to investigate the clinical features and the potential relationship between this genotype and phenotype for the specific mutationc.1663G > A (p.A555T).

Methods

1. **Patients**

From 2004 to 2019, a total of 1799 MMA patients were diagnosed and treated at multiple hospitals in China. Among them, 328 cases were caused by the *MMUT* gene mutation. We searched for cases carrying the mutationc.1663G>A (p.A555T) with in these patients. As a result, a total of 30 patients (9.15%) were collected. We consider them here after as “c.1663G>A group”. To match the patients carrying c.1663 G>A and “another mutation” in another allele, we selected another 36 MMA patients sharing the some “another mutation” with c.1663 G>A group, as paired control group (non-c.1663G>A group). We compared the clinical and biochemical phenotypes of patients from the two groups. Written informed consent was obtained from the parents of study participants. This study was approved by the Ethics Committee of Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (approval ID:XHEC-D-2020-024).

2. **Detection of metabolites**

Blood levels of acylcarnitines, including propionylcarnitine (C3) and acetylcarnitine (C2) were detected by MS/MS (API 4000, American Bio-Systems Inc) using blood filter papers. Urinary organic acids, including methylmalonic acid and methylcitric acid were measured by gas chromatography-mass spectrometry (GC-

MS) (Shimadzu Limited, QP2010).

3. ***MMUT* gene mutation detection and evaluation**

MMUT gene test was performed by Sanger sequencing or high-throughput next generation sequencing. The mutation was identified by the normal human *MMUT* sequence as a reference (GenBank, NC_000006.12). We used the ClinVar database, the HGMD database and the former literatures to identify whether the mutations had been reported. The pathogenicity of the missense mutation was predicted by the Mutation Taster, PolyPhen-2, Proven and SIFT software. Clustal Omega and HOPE website (https://swissmodel.expasy.org) were used to show the position the mutation occurred in the protein. The HOPE website was also used to establish a crystal structure of human MCM protein with mutation, and then evaluated the potential impact of the mutation on the protein structure.

4. **Treatment**

The treatment of MMA varies with different vitamin B12 responsiveness of the patients. Generally, vitamin B12 responsive patients are treated by vitamin B12, L-carnitine, and low isoleucine, valine, threonine, methionine diet, while vitamin B12 unresponsive patients are treated by L-carnitine and the special diet [19]. Being vitamin B12 responsive is defined as a reduction of more than 50% in the C3/C2 and methylmalonic acid content after vitamin B12 loading test, compared with those before treatment. If the blood C3/C2 ratio and urine methylmalonic acid are decreased but less than down to 50% after vitamin B12 loading test, it is deemed to be “partly responsive” [20]. Among these patients, 29 patients underwent vitamin B12 loading test, 28 with hydroxocobalamin and 1 with mecobalamin. All of them were proven to be responsive to vitamin B12. Then, most of the patients were treated by hydroxocobalamin with a dose of 0.025–0.25mg/kg/day. Besides, most of them were also treated by L-carnitine with a dose of 50–100 mg/kg/day.

5. **Statistical analysis**

The normally distributed measurement data were statistically evaluated by using Student t-test, while non-normally distributed data were analyzed by Wilcoxon rank-sum test. The comparison of rates was managed by Chi-square test. Statistical analyses were performed using the GraphPadPrism 5 software (GraphPad Software Inc., San Diego, CA, USA). The p-value of <0.05 was considered as a significant difference between two groups.

Results

1.Clinical features of the patients

1.1 Clinical features of c.1663G>A group

The detailed information on each patient carrying c.1663G>A (p.A555T) mutation is summarized in table 1. Up to now, their median age was 2.8 years old, ranging from 7 months to 14 years old. Among these patients, 26 cases were diagnosed by using a positive newborn screening, 2 patients were diagnosed because of onset of the disease (P18, P30), and 2 cases (P9, P13) were diagnosed because of sibling MMA diagnosis (P8,P12). Only 7 cases encountered disease onset, among which 4 cases were subjected to newborn screening (P15, P19, P24,P29) and 3 cases were not (P9, P18, P30).

1.1.1 Clinical manifestation and treatment

Among the 7 cases with onset, 3 cases showed acute disease onset (P19, P29, P30), while 4 cases developed mental retardation progressively, without acute symptoms (P9, P15, P18, P24). As for the 3 cases with acute disease onset, the symptoms showed no specificity, including difficult feeding, vomiting, diarrhea, muscle weakness, lethargy, and convulsion. The median age of disease onset was 16 months old, ranging from 3 days to 22 months old.

The patient P19 was diagnosed by newborn screening and was treated by L-carnitine since 1 month of age. An acute attack of metabolic acidosis induced by upper respiratory tract infection, showing the symptoms of vomiting, diarrhea, lethargy, and muscle weakness happened at 22 months of age. After symptomatic treatment, the patient gradually recovered in two weeks. P29 had undergone newborn screening at birth. However, the individual did not accept treatment until a disease attack induced by respiratory tract infection at 16 months old, manifested with fever, vomiting, diarrhea, muscle weakness, lethargy, convulsion, and coma. Auxiliary examination indicated metabolic acidosis. P30 was observed to present difficult feeding, poor weight gain, muscle weakness, and metabolic acidosis at 1 month of age.

As for the 3 patients that showed progressive mental retardation, P9 was diagnosed because the younger sibling (P8) was confirmed with MMA in newborn screening, and then, detection of the gene confirmed the diagnosis of P9. A mild mental retardation was observed when diagnosis was confirmed at 23 months old. The patient could not speak until the age of 26 months. The patient P15 showed delayed development and could not walk until the age of 16 months. P18 was not subjected to newborn screening at birth 8 years ago. The individual could not walk until 24 months old, could not speak until 36 months old, and was diagnosed with MMA at 57 months old because of mental retardation. P24 was diagnosed by newborn screening and was treated irregularly. This patient presented progressive developmental delay without attack of metabolic acidosis. Furthermore, the child could not walk properly at the age of 17 months.

1.1.2 Treatment and vitamin B12 responsiveness

All the 30 patients accepted treatment after diagnosis. More than half of patients (16/30) received the treatment before being 2 months old. Most of the patients were treated with L-carnitine and hydroxocobalamin. A few patients accepted one of the two drugs. Nearly half of the patients insisted on the specialized diet therapy. Except for P13 who had not attempt vitamin B12 loading test, all the 29 patients were responsive to vitamin B12, with the responsive rate of 100%. The patient P15 was diagnosed by newborn screening and was adhered to a low protein diet. The vitamin B12 and L-carnitine were added until 1

year old. The patients P19 and P24 were diagnosed by newborn screening and treated with L-carnitine and hydroxocobalamin since the age of about 1 month. L-carnitine was kept at a dose of 100mg/kg/d routinely. The hydroxocobalamin treatment for P24 was stopped by parents without the permission of the doctor when the patient was 2 months old. As for the patient P29, hydroxocobalamin and L-carnitine were used immediately at the disease onset. Of note, this patient recovered quickly and showed no attacks after treatment.

1.1.3 Prognosis

As for the current health condition, following up until December 2019, 2 patients could not be followed up (P20, P30). Twenty-four patients (24/30, 80%) were healthy and lived a normal life asymptomatically. Four patients showed progressive mental retardation (P9, P15, P18, and P24). P15 and P24 were diagnosed in newborn screening while P9 and P18 did not undergo newborn screening. The Gesell developmental schedule scores of P9 at 22 months old were gross motor 86, fine motor 66, adaptive 93, language 44, personal-social 62. Similarly, the Gesell developmental schedule scores of P15 at 25 months old were gross motor 65, fine motor 63, adaptive 58, language 69, personal-social 56. The Gesell developmental schedules scores of P24 at 17months old were gross motor 78, fine motor 69,adaptive 78, language 49 and personal-social 49. After treatment, the intelligence of P18 improved slowly. The WISC developmental schedule scores of this patient at 7.2 years were verbal IQ 44, performance IQ <40, and total IQ<40.

The patients P19 and P29 who had experienced disease onsets display currently normal intelligence after treatment. The diagnosis of MMA was achieved, and vitamin B12 was used when P30 was 2.5 months old. Unfortunately, individual´s parents gave up the treatment at 3 months old and we failed to confirm the current health status of P30.

1.2 Clinical features of non-c.1663G>A group

The detailed information on the enrolled patients of the control group is summarized in table 2. There were 23 boys and 13 girls in the control group, with a median age of 2.9 years old, ranging from 12 months to 12.5 years old. Twenty-two patients missed newborn screening and were diagnosed because of the onset of the disease. Further,14 patients were diagnosed by newborn screening, in which10 patients showed disease onset during their next treatment. The median age for disease onset of these patients was 3 months old. The symptoms were manifested in varied forms, including difficult feeding, vomiting, diarrhea, poor weight gain, muscle weakness, dyskinesia, lethargy, convulsion, coma, mental retardation, jaundice, anemia, metabolic acidosis, and progressive developmental delay. All the 36 patients accepted treatment after diagnosis. Their median age of beginning treatment was 2 months old. Except for 1case lost during the follow up (C17) and 3 cases of death prior to vitamin B12 treatment (C1, C7, and C8), the remaining 32 patients were subjected to the vitamin B12 loading test. It was seen that 12 patients were responsive to vitamin B12, while the other 20 patients were unresponsive to vitamin B12, yielding a total vitamin B12 responsive rate of 38%, which is significantly lower than that for the c.1663G>A group. As for the current health condition under treatment, 24 patients showed mental retardation (67%), 5 patients are living healthy lives asymptomatically, and 6 patients died from disease onset at ages ranging from 7 days to 18 months.

1.3 Comparison of clinical features in two groups

The detailed clinical features comparison of c.1663 G>A group and non-c.1663 G>A group are summarized in table 3. There were significant differences in presentation and clinical severity between the two groups. The proportions of disease onset were 7/30 (23%) in c.1663 G>A groupand 32/36 (89%) in non-c.1663 G>A group, with a significant difference in the incidence rate between the two groups (P< 0.0001). As for the treatment, the vitamin B12 responsive rate was 100% (29/29) in c.1663G>A group and only 38% (12/32) in non-c.1663G>A group (P< 0.0001). As for the prognosis, most of the patients carrying c.1663G>A (p.A555T) remained asymptomatic under treatment (24/30). In contrast, most of the patients carrying other mutations manifested developmental retardation (24/36), and 6 patients died. A significant difference was also detected in the prognosis of the two groups (P< 0.0001). In conclusion, compared with patients carrying other mutations, c.1663G>A (p.A555T) - coding patients exposed lower morbidity, later disease onset, milder clinical phenotype, better vitamin B12responsiveness, and thereby better prognosis.

2. **Biochemical features of the patients**

As the biochemical makers, the blood C3, blood C3/C2 ratio, urinary methylmalonic acid, urinary methylcitric acid before and after treatment in c.1663G>A and non-c.1663G>A groups are presented in table 1 and table 2. The comparative results between c.1663G>A and non-c.1663G>A groups are summarized in table 3.

On the primary state before treatment, C3, C3/C2, methylmalonic acid, methylcitric acid in c.1663G>A group showed a slight increase over the norm range. In contrast, the 4 biochemical indexes of the non-c.1663G>A group showed a prominent increase than the normal range in most patients.

All the 4 biochemical markers in c.1663G>A group before treatment were much lower than those in non-c.1663G>A group, with a significant statistical difference (table1, table 2, table 3). Similar changes were observed in the two groups after the treatment. The levels of C3, C3/C2 ratio, methylmalonic acid, methylcitric acid in c.1663G>A group after treatment were much significantly lower than those in non-c.1663G>A group. As for c.1663G>A (p.A555T) carrying patients, the levels of C3, C3/C2 ratio, methylmalonic acid, methylcitric acid decreased remarkably after treatment, compared with those before treatment. However, in non-c.1663G>A group, C3, methylcitric acid decreasedwhile C3/C2 ratio, methylmalonic acid increased after treatment, compared with those before treatment, respectively (table1, table 2, table 3). These data indicated that the therapeutic effect in c.1663G>A (p.A555T) carrying patients was much better than that innon-c.1663G>A (p.A555T) carrying patients.

3. **Geographical distribution of c.1663G>A (p.A555T)**

In order to explore the geographical distribution of the mutation c.1663G>A (p.A555T), we analyzed the originof the 328 patients harboring mutations in the *MMUT* gene, and calculated the mutation frequency of c.1663G>A (p.A555T) in different regions. As shown in table 4, the mutation frequency varies notably depending on the region. The population of the Shandong province was found to display the highest mutation frequency, followed by Hebei province and Henan province.

4. **Pathogenic effects of mutation c.1663G>A (p.A555T)**

We assessed the potential pathogenicity of the mutationc.1663G>A (p.A555T) by MutationTaster, PolyPhen-2, Proven and SIFT software. It was predicted to be “disease-causing”, “probably damaging”, “deleterious”, and “damaging”, respectively. Furthermore, we evaluated its pathogenicity by the WinterVar database (http://wintervar.wglab.org), according to the ACMG 2015 guideline. It was defined as “likely pathogenic” with the score “PM1+PM2+PP3+PP5”.

The mutationc.1663G>A (p.A555T) leads to analanine into a threonine change at position 555 in the MCM protein. The website “HOPE” (https://www3.cmbi.umcn.nl/hope) was used to model the conceivable 3D conformations of wild-type and mutant MCM proteins, which are illustrated in figure 1. Figure1A shows the schematic structures of the origin alanine and the mutant threonine. The mutant threonine residue is larger and less hydrophobic than the wild-type alanine residue. The wild-type residue 555, colored in green in figure 1B, is located in an α-helix ranging from amino acid 548 to 557, and is buried in the core of the protein. The mutated residue threonine, colored in red in figure 1C, does not prefer α-helices as a secondary structure for the steric hindrance effect and the loss of hydrophobic interactions, and thereby affects the function of the protein, as shown in figure 1D [21].

Discussion

The *MMUT* gene harbors wide variety of mutations and differs significantly in different races. The frequency of c.1663G > A (p. A555T) is considered to be an extremely rare mutation. In China, we found it to be the most frequently occurring in the Shandong, Hebei, and Henan provinces. Each amino acid has its own specific size, charge, and hydrophobicity. The original wild-type residue and mutant residue often differ in properties. The amino acid site 555 is highly conserved. There were no other residues observed at this position in other homologous sequences. Missense mutations occurred at nearby positions (A535P, C560Y) have been reported in association with MMA. Mutation Taster, PolyPhen-2, Proven, SIFT software and ACMG guideline predicted c.1663G > A (p.A555T) to be pathogenic or probably pathogenic mutation. Besides, the mutation was defined as pathogenic in the Clinvar database based on the reported patient [17]. Furthermore, the 3D-structure model supported the potential impact of the mutation on the crystal structure of the MCM protein. In conclusion, the mutationc.1663G > A (p.A555T) tends to impair the function of MCM protein and cause the disease.

Most of the patients in our cohort were diagnosed by newborn screening without symptoms, and a few were clinically confirmed upon onset of the disease. All of them were responsive to the treatment with vitamin B12, and most of them retained asymptomatic thereafter. We suggest that c.1663G > A (p.A555T)-carrying patients caused milder clinical phenotype, better vitamin B12 responsiveness, and better prognosis. There is no specificity in the clinical manifestations of these patients on disease onset, such as vomiting, diarrhea, metabolic acidosis. Despite the milder presentation, complications such as mental retardation may also occur, especially if treatment is delayed, such as that observed in case of P9, P15, and P18 in our cohort. MS/MS, GC-MS, and gene detection are critical diagnostic methods for the disease and should be promoted in clinically doubtful patients. Early diagnosis and treatment are important even in individuals with milder, presumably late-onset disease. Newborn screening plays critical role in the early diagnose of the disease and should be promoted for a broader range of newborns in China to detect this disease.

As for the biochemical phenotype, patients carrying c.1663G > A (p.A555T) had slightly elevated blood C3, blood C3/C2 ratio, urinary methylmalonic acid, urinary methylcitric acid, which were much lower than those of non-c.1663G > A carrying patients, both before and after treatment. These biochemical markers in c.1663G > A group showed a more pronounced decrease than non-c.1663G > A group during the treatment, also suggesting a better therapeutic effect in c.1663G > A carrying patients. Because of the mild biochemical phenotype, the clinical manifestation is also correspondingly mild. For these patients, gene sequencing is a key assessment in confirming the diagnose which should be performed in time.

At present, there is no information on the potential underlying mechanism explaining why the mutation c.1663G > A (p.A555T) causes the milder phenotype. One consideration is based on the crystallography of the protein MCM. The MMUT mRNA transcript encodes 750 amino acids, which constitute the immature enzyme. Upon entering the mitochondria, the mitochondrial leader sequence (residues 1–32) is cleaved from the immature MCM. Mature human MCM is a homodimer. Each subunit contains an N-terminal extended segment (residues 33–87), an N-terminal (βα) 8 TIM barrel domain (residues 88–422), a linker region (residues 423–577) and a C-terminal (βα) 5 Rossmann domain (residues 578–750)[18]. The N-terminal extended segment is involved in subunit interaction and precedes two functional domains of the protein. The N-terminal (βα) 8 TIM barrel domain contains the substrate-binding site. The C-terminal (βα) 5 Rossmann domain is the adenosylcobalamin binding domain. The two functional domains are connected by the linker region, in which the mutation c.1663G > A (p.A555T) is located. Being different from the mutations positioned in the catalytic domain and binding domain, the mutations located in the linker region have relatively little effect on the protein function. A 17-month-old Hmong MMA patient carrying homozygous c.1663G > A (p.A555T) mutation in the *MMUT* gene has been mentioned by Jordan Chu et al [18]. In this study, the authors defined the patient to mut^- type instead of mut^0 type, but there was no further clinical description of the patient. Besides, in a large-scale evaluation of molecular genetic characterization of 151 mut^- type MMA patients by Forny P, deficient alleles in the mut^- subclass were almost exclusively caused by missense mutations, found disproportionately in the C-terminal cofactor binding domain. On the contrary, only half of the mut^0genotypes were of the missense type. Western blot analysis revealed protein instability as a major mechanism of deficiency in mut^- type MMA[1]. As for the *MMUT* mutations and their relationship to dysfunction and disease, we assume that the milder phenotype induced by c.1663G > A (p.A555T) might also benefit from a missense type of mutation, causing an amino acid substitution rather than deletion of a section of amino acids. Furthermore, the human *MMUT* gene is located on chromosome 6p12.3 and spans over 13 exons. The mutation c.1663G > A (p.A555T) is located in exon 9, which is relatively near to the C-terminal (555/750), which is considered to elicit less effect on the protein functions. It might be another reason for the lighter phenotype caused by the mutation.

Three cases (P28, P29, and P30) in our cohort had the classical clinical and biochemical manifestation of MMA. However, we failed to identify their another mutation by DNA sequencing. Two of the 3 patients had encountered the disease onset at 16 months (P29) and 1 month old (P30) with severe symptoms, such as muscle weakness, vomiting, lethargy, convulsion and even coma, which suggested that variations existing in their another allele might seriously affect protein function. It is well known that the deletion of exon generally often leads to a more severe phenotype. Therefore, we speculate that exon deletion might exist on another allele in these patients.

Conclusion

Most of our patients carrying c.1663G > A (p.A555T) were diagnosed through the newborn screening, were timely treated, and kept asymptomatic until now. MMA patients with the mutation c.1663G > A (p.A555T) display mild clinical and biochemical phenotype, which was indicated by distinct lower morbidity, later disease onset, milder clinical phenotype, lighter biochemical abnormalities, better vitamin B12 responsiveness, easier metabolic control, and better prognosis. Meanwhile, mild phenotype makes the disease more likely to be missed. Thus, gene sequencing and newborn screening by MS/MS facilitate early diagnosis and treatment for the disease and should be promoted further.

Abbreviations

GC-MS: gas chromatography-mass spectrometry; MCM: methymalonyl-CoA mutase; MMA: MethylImalonic academia; MS/MS: tandem mass spectrometry.

Declarations

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Availability of data and materials

All data generated or analysed during this study are included in the published article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine (Approval number: XHEC-D-2020-024).

Consent for publication

Participants provided written informed consent.

Competing interests

The authors declare that there is no conflict of interest about this article.

Authors' contribution:

Dr. Lili Liang, as the doctor of many of these patients, contributes to reorganize and analyze of the clinical data of the patients and draft the manuscript.

Dr. Ruixue Shuai, contributes to collect and reorganize the clinical data of the patients, and revise the manuscript.

Dr.Yue Yu contributes to collect the clinical data of the patients.

Dr.Wenjuan Qiu, Dr. Linghua Shen, Dr. Shengnan Wu, Dr. Haiyan Wei, Dr. Yongxing Chen, Dr. Chiju Yang, Dr. Peng Xu, Dr. Xigui Chen, Dr. Hui Zou, Dr. Jizhen Feng, Dr. Tingting Niu, Dr. Haili Hu, Dr. Jun Ye, Dr. Huiwen Zhang, Dr. Deyun Lu, Dr. Yongguo Yu, and Dr.Xuefan Gu contribute to collect and treat the patients and provide the clinical data.

Mrs. Wenjun Ji contributes to detection the blood acylcarnitines of the patients' by tandem mass spectrometry.

Mrs. Xia Zhan contributes to detection the urinary organic acids of the patients' by gas chromatography-mass spectrometry.

Mrs. Zhuwen Gong contributes to gene variation analysis.

Dr. Lianshu Han, as the doctor of most of the patients, contributes to design the research, treat the patients, provide the clinical data and revise the manuscript.

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Tables

Table 1. Clinical characteristics of the patients carrying c.1663G>A (p.A555T)

Case No.	Sex	Age	NS	Age of beginning to Treat	Disease Onset	Age at Onset	Current Health Condition	Hydroxocobalamin effective	On Presentation				After treatment				Mutation 1	
									C3	C3/C2	Methylmalonic acid	Methylcitric acid	C3	C3/C2	Methylmalonic acid	Methylcitric acid		
P1	F	13 ys	yes	1 m	no	/	healthy	yes	7.92	0.69	566.23	4.38	3.29	0.26	22.40	1.27	c.1663G>A, p.A555T	
P2	M	5 ys	yes	4 ms	no	/	healthy	yes	6.06	0.31	2.31	1.92	3.88	0.23	4.55	1.75	c.1663G>A, p.A555T	c.7
P3	M	3 ys	yes	3 ms	no	/	healthy	yes	4.89	0.33	4.62	/	2.967	0.16	0	0.27	c.1663G>A, p.A555T	c.7
P4	F	3 ys	yes	1 m	no	/	healthy	yes	6.98	0.69	84.50	/	6.781	0.354	47	1.1	c.1663G>A, p.A555T	c.7
P5	M	1 y	yes	3.7 ms	no	/	healthy	yes	4.95	0.32	18	1.08	2.51	0.08	9.44	0	c.1663G>A, p.A555T	c.7
P6	M	11ms	yes	1 m	no	/	healthy	yes	7.88	0.49	27	1.1	8.52	0.16	30	1.62	c.1663G>A, p.A555T	c.7
P7	M	1 y	yes	1 m	no	/	healthy	yes	3.8	0.52	228.11	9.68	1.83	0.1	2.1	0	c.1663G>A, p.A555T	c.7
P8	F	2.5ys	yes	1 m	no	/	healthy	yes	6.67	0.69	94.31	/	2.20	0.17	0.90	/	c.1663G>A, p.A555T	
P9	M	4 ys	no	23 ms	yes	PDD	retardation	yes	3.86	0.34	6.2	0	3.3	0.25	0.3	0	c.1663G>A, p.A555T	
P10	F	4 ys	yes	1.7 ms	no	/	healthy	yes	7.11	0.72	124.80	/	2.15	0.28	2.06	0.36	c.1663G>A, p.A555T	
P11	F	8 ms	yes	1 m	no	/	healthy	yes	5.82	0.18	20.09	2.2	1.31	0.07	1.32	0.56	c.1663G>A, p.A555T	
P12	M	7 ms	yes	1 m	no	/	healthy	yes	4.63	0.23	34.6	1.4	1.903	0.113	18.9	/	c.1663G>A, p.A555T	
P13	F	3.4ys	no	3ys	no	/	healthy	not used	3.97	0.27	5.7	0.8	6.3	0.22	15	0.9	c.1663G>A, p.A555T	
P14	F	3 ys	yes	2 ms	no	/	healthy	yes	5.02	0.38	/	/	3.8	0.2	/	/	c.1663G>A, p.A555T	
P15	F	4 ys	yes	1y	yes	PDD	retardation	yes	9.72	0.51	38.96	1.2	7.17	0.26	4.28	0.53	c.1663G>A, p.A555T	
P16	M	3 ys	yes	1.3 ms	no	/	healthy	yes	4.86	0.37	9.27	1.33	2.188	0.09	0	0.11	c.1663G>A, p.A555T	
P17	M	2 ys	yes	1.3 ms	no	/	healthy	yes	8.65	0.7	133.91	2.26	5.87	0.2	/	/	c.1663G>A, p.A555T	
P18	M	8 ys	no	4.5ys	yes	PDD	retardation	yes	3.01	0.19	43.77	/	2.46	0.11	1.1	0	c.1663G>A, p.A555T	
P19	F	3.5ys	yes	1 m	yes	1.8y	healthy	yes	7.81	0.92	100.08	3.78	5.42	0.3	3	0.13	c.1663G>A, p.A555T	
P20	M	4.2ys	yes	4 ms	no	/	loss of follow up	yes	4.13	0.69	63.82	0.42	4.30	0.30	85.17	4.2	c.1663G>A, p.A555T	c.
P21	M	3 ys	yes	1.4 ms	no	/	healthy	yes	6.76	0.62	286.19	4.22	2.92	0.06	12.77	1.01	c.1663G>A, p.A555T	
P22	M	2. ys	yes	1 m	no	/	healthy	yes	6.54	0.33	92.69	2.58	1.96	0.06	13.4	0.6	c.1663G>A, p.A555T	
P23	F	1. ys	yes	4 ms	no	/	healthy	yes	5.94	0.45	43.25	/	2.384	0.149	3.25	0	c.1663G>A, p.A555T	c.
P24	M	1.6ys	yes	1.3 ms	yes	PDD	retardation	yes	5.85	0.39	22.31	1.5	7.26	0.15	2.93	0.63	c.1663G>A, p.A555T	
P25	F	2.6ys	yes	20 ds	no	/	healthy	yes	4.1	0.34	8.5	0	7.41	0.2	33	1.1	c.1663G>A, p.A555T	
P26	F	2.5ys	yes	2 ms	no	/	healthy	yes	2.4	0.32	45.6	1.07	1.78	0.1	0	0	c.1663G>A, p.A555T	
P27	F	1. ys	yes	1 m	no	/	healthy	yes	5.82	0.30	28.80	2.16	4.426	0.133	3.79	0	c.1663G>A, p.A555T	
P28	M	2 ys	yes	1 m	no	/	healthy	yes	4.71	0.41	29.7	0.7	4.93	0.2	77.7	2	c.1663G>A, p.A555T	
P29	M	2 ys	yes	16 ms	yes	16 ms	healthy	yes	4.67	0.34	174.7	0.89	3.16	0.13	7.25	0	c.1663G>A, p.A555T	
P30	M	14ys	no	2.5 ms	yes	1 m	loss of follow up	yes	6.55	0.85	/	/	1.85	0.31	/	/	c.1663G>A, p.A555T	

M:male; F:female; NS: Newborn Screening; PDD: progressive developmental delay; y: year; m:month; d:day

Typical reference range of C3:0.50-4.00μmol/L; Typical reference range of C3/C2:0.04-0.25;

Typical reference range of methylmalonic acid :0-4mmol/mol creatinine; Typical reference range of methylcitric acid:0-0.8 mmol/mol creatinine.

Table 2. Clinical characteristics of the patients in control group

CaseNo.	Sex	Age	NS	Age of Beginning to Treat	Disease Onset	Age at Onset	Current Health Condition	Hydroxocobalamin effective	On Presentation				After treatment				Mu
									C3	C3/C2	Methylmalonic acid	Methylcitric acid	C3	C3/C2	Methylmalonic acid	Methylcitric acid	
C1	M	/	no	5ds	yes	3ds	died (7ds)	unevaluated	13.33	1.00	301.36	63.64	/	/	/	/	c.729_730ins
C2	M	2ys	no	7ds	yes	3ds	retardation	no	28.74	1.54	274.80	14.10	41.67	1.28	1937.12	60.23	c.729_730ins
C3	M	4.1ys	yes	1m	yes	3ds	retardation	no	23.42	1.02	253.00	17.00	32.81	0.66	195.40	0.87	c.729_730ins
C4	M	2.9ys	yes	1m	no	/	healthy	yes	6.20	0.32	62.00	1.58	7.30	0.37	34.60	2.00	c.729_730ins
C5	M	2ys	no	20ds	yes	3ds	retardation	no	8.68	0.43	258.90	19.80	34.18	1.23	1062.27	30.91	c.729_730ins
C6	M	2.8ys	no	15ms	yes	3ms	retardation	yes	7.80	0.26	319.00	41.80	21.90	0.47	130.00	4.50	c.729_730ins
C7	M	/	no	3ds	yes	3ds	died (10ds)	unevaluated	19.46	1.30	106.43	8.73	/	/	/	/	c.729_730ins
C8	M	/	no	2ds	yes	3ds	died (7ds)	unevaluated	15.01	1.45	2541.20	84.00	/	/	/	/	c.729_730ins
C9	F	12ys	no	20ms	yes	1.6ys	retardation	yes	/	/	1426.29	15.33	52.19	0.93	0.00	0.30	c.1106G>
C10	F	7ys	no	12ms	yes	12ms	retardation	no	15.92	1.03	869.83	10.09	12.75	0.74	701.72	8.56	c.1106G>
C11	F	/	no	2.5ms	yes	3ds	died (17ms)	no	10.93	2.10	402.98	40.68	18.96	0.96	0.00	33.41	c.1106G>
C12	F	4ys	no	16ms	yes	16ms	retardation	no	43.71	0.60	7202.00	/	20.71	0.77	1068.00	5.51	c.1106G>
C13	M	2.6ys	yes	2ms	yes	17ms	retardation	no	9.46	0.82	448.50	16.20	18.12	0.54	867.31	5.87	c.1106G>
C14	F	2ys	yes	20ds	yes	1y	healthy	no	10.32	0.54	704.12	12.30	47.88	0.89	354.51	3.17	c.1106G>
C15	F	1.5ys	yes	7ds	yes	3ds	retardation	yes	18.00	1.10	523.23	5.52	39.07	1.40	382.44	5.40	c.1106G>
C16	M	1.5ys	yes	1.3ms	yes	PDD	retardation	no	21.10	0.79	/	/	29.94	0.95	1929.39	47.28	c.2131G
C17	M	2ys	yes	2ms	no	/	loss of follow up	unevaluated	2.16	0.37	36.97	3.26	/	/	/	/	c.2131G
C18	M	7.5ys	no	8ms	yes	6ms	retardation	no	4.76	0.36	/	/	26.68	0.53	790.1	3.1	c.2131G
C19	M	5.5ys	no	7ds	yes	3ds	retardation	yes	11.81	0.43	9.83	0.00	17.68	0.67	103.00	/	c.424A>
C20	F	1y	yes	3ms	yes	7ds	retardation	no	7.92	8.81	/	/	63.32	1.06	/	/	c.424A>
C21	M	9.5ys	no	7ms	yes	7ds	retardation	no	13.57	0.47	286.85	/	24.77	0.85	162.51	/	c.424A>
C22	F	1.9ys	yes	1m	no	/	healthy	yes	9.00	1.80	10.90	5.58	24.83	0.44	263.77	0	c.494A>
C23	M	3.3ys	no	7ds	yes	3ms	died (18ms)	no	15.70	0.66	409.00	6.93	14.65	0.62	617.71	22.59	c.494A>
C24	F	13ys	no	46ms	yes	46ms	healthy	no	13.19	0.50	/	/	18.12	0.99	256.78	1.17	c.613G>
C25	M	2.5ys	yes	20ds	yes	6ms	retardation	no	10.87	0.66	14.85	8.39	32.18	0.84	450.46	4.04	c.613G>
C26	M	/	no	2ms	yes	3ds	died (5ms)	no	10.33	0.72	422.84	/	34.03	1.06	495.00	/	c.626du
C27	M	4.8ys	no	17ms	yes	1y	retardation	no	21.77	0.53	453.76	10.21	12.15	0.99	145.40	5.54	c.626du
C28	M	2.5ys	no	6ms	yes	3ms	retardation	yes	18.53	0.55	130.60	12.70	32.09	0.88	0.64	14.80	c.755_756ins
C29	M	6ys	no	12ms	yes	12ms	retardation	yes	4.70	0.38	278.00	1.70	33.07	1.08	1334.22	9.44	c.914T>
C30	F	4.5ys	no	6ms	yes	5ms	retardation	no	4.83	0.51	422.73	2.89	16.86	0.91	1254.17	30.46	c.914T>
C31	F	2.4ys	yes	1m	yes	15ms	retardation	no	13.50	1.53	431.92	22.45	32.02	1.41	467.17	8.53	c.914T>
C32	F	3ys	yes	2ms	no	/	healthy	yes	15.45	0.22	180.70	/	6.08	0.21	116.03	2.96	c.1233_1235
C33	M	4ys	no	4ms	yes	4ms	retardation	no	6.66	0.76	165.30	4.00	12.24	0.81	146.72	0.75	c.1280G
C34	M	1.6ys	yes	10ds	yes	7ds	retardation	yes	13.61	0.85	284.80	21.20	30.22	1.08	744.63	5.94	c.1280G
C35	F	1.8ys	yes	10ds	yes	7ds	retardation	yes	9.81	1.08	11057.34	41.93	27.18	1.42	520.08	5.16	c.1679G>
C36	M	3ys	no	16ms	yes	8m	retardation	yes	26.37	0.59	332.80	0.7	26.62	0.43	335.00	1.80	c.2009G>

M:male; F:female; NS: Newborn Screening; PDD: progressive developmental delay; y: year; m:month; d: day

Typical reference range of C3:0.50-4.00μmol/L; Typical reference range of C3/C2:0.04-0.25;

Typical reference range of methylmalonic acid :0-4mmol/mol creatinine; Typical reference range of methylcitric acid:0-0.8 mmol/mol creatinine.

Table 3. Patient cohort characteristics.

Variable Value	c.1663 G>A group	non-c.1663 G>A group (n = 36)	Statistical difference
Number of subjects	n = 30	n = 36	
Age (year)	0.58-14.00 (mean 3.44; median 2.80)	1.00-12.5 (mean 3.99; median 2.90; n=31, the remained 5 cases died)	
Male: female	17/13	23/13	
Newborn screening	26/30 (87%)	14/36 (39%)	
Disease Onset	7/30 (23%)	32/36 (89%)	$\chi2=$ 29.09; $P<$ 0.0001
Average age of acute onset (month)	0.10-22 (mean 13.00; median 16.00, n=3)	0.10-46.00 (mean 6.65; median 3.00; n=30)	
Age of begin treatment (month)	0.70-57 (mean 6.28; median 1.35, n=30)	0.07-46.00 (mean5.77; median 2.00; n=36)	
Vitamin B12 effectiveness	29/29 (100% effective; 1 case has not used Vitamin B12)	12/32 (38% effective; 1 case was lost of followed up and could not be evaluated; 3 cases died before Vitamin B12 use)	$\chi2=$ 26.97; $P<$ 0.0001
Current Health Condition	healthy: 24 cases; mental retardation:4 cases; loss of follow up: 2 cases.	mental retardation:24 cases; died: 6 cases; healthy:5 cases; loss of follow up: 1 cases.	$\chi2=32.79$; $P<$ 0.0001
C3 level on presentation (μmol/L)	median 5.82 (2.40–9.72; n=30)	median 13.19 (2.16–43.71; n=35)	U=121.50; $P<0.0001$
C3/C2 level on presentation	median 0.39 (0.18-0.92; n=30)	median 0.66 (0.22-8.81; n=35)	U=257.50; $P=0.0004$
Methylmalonic acid level on presentation (mmol/mol creatinine)	median 41.11 (2.31-566.20; n=28)	median 310.20 (9.83-11057.00; n=32)	U=132.00; $P<0.0001$
Methylcitric acid level on presentation (mmol/mol creatinine)	median 1.37 (0.00-9.68;n=22)	median 11.26 (0-84.00;n=28)	U=73.50; $P<0.0001$
C3 level after treatment (μmol/L)	median 3.23 (1.31-8.52 ; n=30)	median 26.65 (6.08-63.32; n=32)	U=8.00; $P<0.0001$
C3/C2 level after treatment	0.18±0.02 (0.06-0.35; n=30)	0.86±0.06 (0.21-1.42; n=32)	T=11.56; $P<0.0001$
Methylmalonic acid level after treatment (mmol/mol creatinine)	median 4.28 (0.00-85.17; n=27)	median 382.40 (0.00-1937.00; n=31)	U=77.00; $P<0.0001$
Methylcitric acid level after treatment (mmol/mol creatinine)	median 0.53 (0.00-4.20; n=25)	median 5.46 (0.00-60.23; n=28)	U=65.50; $P<0.0001$

Typical reference range of C3:0.50-4.00μmol/L; Typical reference range of C3/C2:0.04-0.25;
Typical reference range of methylmalonic acid :0-4mmol/mol creatinine; Typical reference range of methylcitric acid:0-0.8 mmol/mol creatinine.

Table 4. The mutation frequency of c.1663G>A (p.A555T) in different regions in the conhort.

Province	Number of cases carrying c.1663G>A	Number of cases caused by MMUT gene mutation	Variation frequency of c.1663G>A
Shandong	14	88	14/176 (7.95%)
Henan	7	56	7/112 (6.25%)
Hebei	3	19	3/38 (7.89%)
Jiangsu	2	24	2/48 (4.17%)
Anhui	1	20	1/40 (2.50%)
Zhejiang	1	19	1/38 (2.63%)
Shanghai	1	13	1/26 (3.85%)
Yunnan	1	1	/
Others districts	0	88	/

Figures

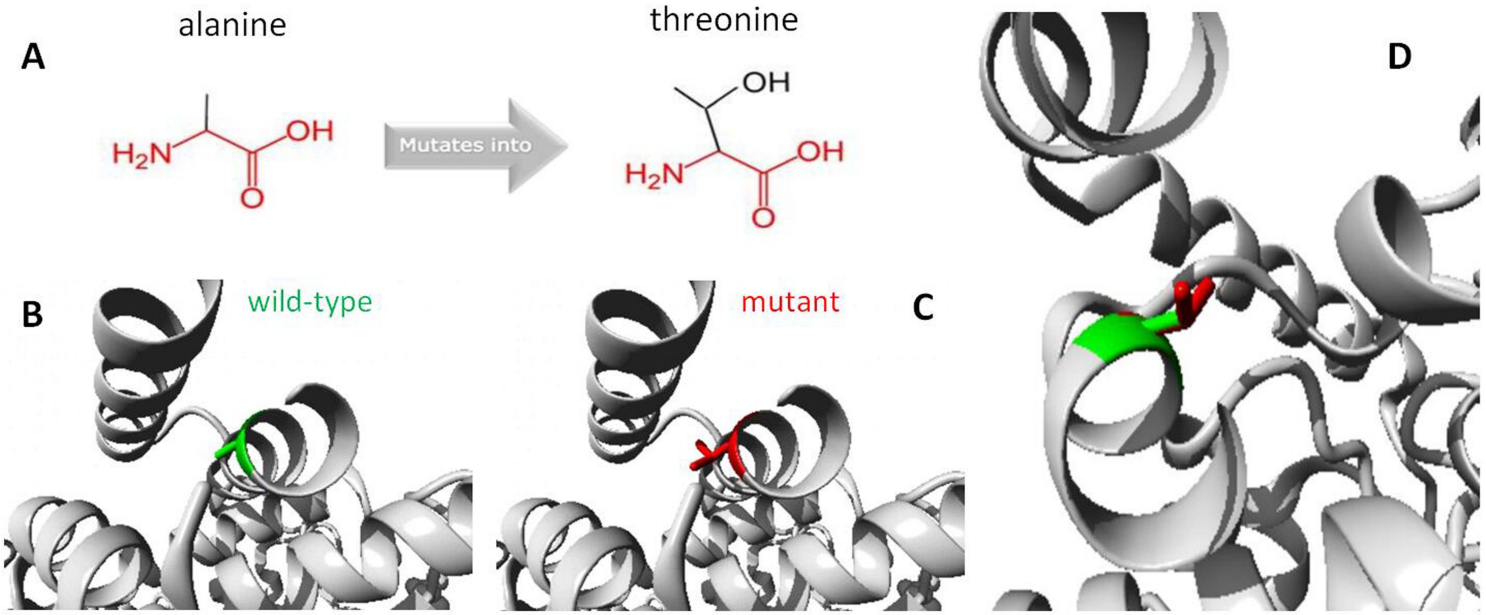

Figure 1

(A) Schematic structures of the wild-typealanine and the mutant threonine. (B) Position of the wild-type residue alanine at position 555, colored in green, in the MCM protein. (C) Position of the mutant residue threonineat position 555, colored in red, in the MCM protein. (D) Modelof the 3D conformations of wild-type and mutant MCM proteins.