Clinical and Molecular Genetic Analysis with Methylmalonic Acidemia Combined with Homocystinuria

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SUMMARY

Background: Based on research, c.609G>A (p.W203X) is a universal mutation site for MMACHC in methylmalonic acidemia (MMA) combined with homocystinuria, cblC type (cblC disease), and c.467G>A (p.G156D) mutation in families with such disease have not yet been reported. To conduct clinical and molecular genetic analysis of a family with cblC disease.

Methods: This work followed the Declaration of Helsinki. All testing methods were performed under the informed consent of our children patients’ parents. A second-generation cblC family with 5 members, was selected as the research subject, including sick siblings and parents and an older sister with normal phenotype, given newborn screening for acylcarnitine spectrum via liquid chromatography tandem mass spectrometry (LC-MS/MS), and diagnosed through combining urine organic acid with homocysteine detection via gas chromatography-mass spectrometry (GC-MS) with second-generation gene sequencing technology. The peripheral blood of five family members was collected for genomic DNA extraction, and the changes were screened in disease-related MMACHC sequence via PCR and direct DNA sequencing.

Results: The family conformed to the autosomal recessive inheritance, the proband and younger sister were cblC patients, diagnosed in February and at 22d given relevant treatment. The proband died, whereas the younger sister received follow-up treatment. Their parents and sister had normal phenotype. In 2 cases, there was compound heterozygous mutation in MMACHC called c.609G>A (p.W203X) nonsense mutation and c.467G>A (p.G156D) missense mutation in exon 4, while the father with normal phenotype had heterozygous mutation c.609G>A in exon 4 coding area. In its protein, the 203rd amino acid changed from tryptophan to a stop codon (p.W203X). The normal mother and sister had a heterozygous mutation c.467G>A in exon 4 coding area. In its protein, the 156th amino acid changed from glycine to aspartic acid (p.G156D).

Conclusions: The cblC family results from c.609G>A (p.W203X) and c.467G>A (p.G156D) compound heterozygous mutations in MMACHC, which has a pathogenic impact.


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KEYWORDS

methylmalonic acidemia, homocystinuria, gene mutation, inheritance

INTRODUCTION

Methylmalonic acidemia (MMA) is the most universal congenital organic acidemia. It is mostly autosomal recessive inheritance with little concomitant inheritance.
Due to the methylmalonyl-CoA mutase (MCM) or cobalamin C (Cbl-C) defect, it results in abnormal accumulation of methylmalonic acid, 3-hydroxypropionic acid, and methyl citrate and other metabolites, affecting homocysteine metabolism [1-3]. MCM is allocated into complete mutase deficiency (mut°) and partial mutase deficiency (mut+) in accordance with the defect degree [4]. Cbl-C defect includes five types: two synthetic defects for adenosylcobalamin (AdoCbl), namely mitochondrial cbl reductase (cblA) deficiency and mitochondrial cbl adenosyltransferase (cblB) deficiency, and 3 synthetic defects (cblC, cblD, cblF) [5] for AdoCbl and methylcobalamin (MeCbl) resulting from abnormal metabolism of cytoplasm and lysozyme cbl. The corresponding coding genes of cblA, cblB, cblC, cblD and cblF are MMAA, MMAB, MMACHC, MMADHC and LBBD1. Mut0, mut-, cblA and cblB only manifest as MMA, so called isolated MMA, whereas cblC, cblD and cblF result in AdoCbl and MeCbl synthesis defects, and elevated homocysteine leads to so-called MMA combined with homocystinuria [6-7].

Based on the literature, the cblC defect caused by MMACHC disorder is the main reason for MMA combined with homocystinuria, the most universal organic acidemia typing in China. Its mutation is available to result in cblC type MMA (cblC disease), which has autosomal recessive inheritance [8]. Based on the literature of European and American countries, the most universal pathogenic mutations of cblC type MMA are c.271dupA and c.331C>T [9]. But c.609G>A mutation is most common in China. MMA combined with homocystinuria cblC type (cblC disease) happens more often in infancy. In China few reports have the detailed clinical data of family and its MMACHC testing. So far, no relevant reports have discussed c.467G>A (p.G156D) mutation in MMACHC with cblC disease. A retrospective analysis of the clinical and laboratory results of a second-generation, 5-member cblC family is being conducted to probe into the pathogenic impact of c.467G>A (p.G156D) and c.609G>A (p.W203X) compound heterozygous mutations in MMACHC.

MATERIALS AND METHODS

General information

This study investigated a second generation family of five without any close relatives. The LC-MS/MS primary screening for detection of the proband and younger sister in the neonatal period was positive. They were recalled to review, and their diagnosis was confirmed through combining GS-MS detecting organic acid in urine and homocysteine with second-generation gene sequencing technology. The five members were genetically analyzed and all tests were conducted under informed consent from the children patients’ parents. The family picture of the child patient was in Figure 1.

The proband II-1 was a boy, 4th child, and 2nd birth. He was delivered by caesarean section due to intrauterine distress under full term, birth weight 3.75kg, with no early broken fetal membrane, abnormalities in amniotic fluid, placenta, and umbilical cord, as well as denying history of rescue for asphyxia. The mother had good maternal health, not consanguineous marriage, denying family genetic history. The first child is a full-term birth of a healthy girl. After the birth, the parents found that children cried and moved less with inflexible eyes, but not treated. Shortly after birth due to vomiting accompanied by fever, the baby went to the neonatology department in our hospital. In accordance with the diagnosis of neonatal infection and hyperbilirubinemia, the baby was given relevant treatment. But with repeated hyperbilirubinemia during hospitalization, the family members did not cooperate with the treatment and signed to leave hospital. Due to the LC-MS/MS primary screening for the baby 11 days after the birth, MMA was suspected. At 46 days following birth, the baby was recalled to review, still with suspected MMA. Therefore, the parents were asked to do regular review of the baby and it was recommended undergo further genetic diagnosis, but they refused. Then, 76 days after birth with a cough for 4 days, the baby was admitted into serious pediatrics of our hospital. The physical examination findings when admitted to hospital: T: 35.9℃, P: 150 times/minute, R: 55 times/minute, wt: 4.7 kg, sable, poor spirit, and nutrition, no rash in the whole body, no touchable swelling in ear, neck and other local shallow lymph nodes, short breath, irregular breathing, three depression sign (+), pharynx congestion, soft neck, coarse sound in breathing with double lung, wet tone, strong and rhythm heart tone, no murmur, soft abdomen, no touchable clots. The part 3 cm under the right rib of liver was soft, not touching the part under the ribs of spleen. The limbs moved freely and hands and feet were warm. On the feet and ankles dark red patterns could be seen but faded when pressed. The recharge time of capillaries was less than 2 seconds. External hospital-assisted examination findings when admitted to hospital: chest x-ray prompted bronchial pneumonia. Blood routine: white blood cells: 3 × 10^9/L; Red blood cells: 2.1 × 10^12/L; hemoglobin: 68 g/L; platelets: 255 × 10^12/L. Liver function, biochemistry, lipids, myocardial enzymes: glutamate transaminase (P): 106 U/L; glutamic oxaloacetic transaminase (P): 106 U/L; creatine kinase (CK) (P): 70 U/L; CK isoenzyme (P): 44 U/L; lactic acid dehydrogenase (P): 448 U/L. The residual findings were generally normal. After admission, the baby received invasive mechanical ventilation, anti-infection, liver protection, C globulin, enhanced support treatment, but repeated hyperbilirubinemia, the baby was given relevant treatment. With bilateral intra...
ventricular hemorrhage, the baby was transferred to the Jinan Children’s hospital to complete cranial CT by the parents themselves, which prompted complex and severe hydrocephalus combined with periventricular edema. Therefore, the mega cisterna magna and arachnoid cyst were considered, maybe with persistent Blake pouch cyst. After receiving invasive mechanical ventilation, anti-infection, levocarnitine, mecobalamin, and other symptomatic treatments, 1 day after improving back to our hospital. After receiving GS-MS to test methylmalonic acid and homocysteine levels as well as genetic testing, he was diagnosed with MMA combined with homocysteine (cblC type) and continued to be given vitamin B12 1 mg/d via intramuscular injections, levocarnitine 200 mg/d via intravenous drip for treatment. Because the child patient before diagnosis had functional damage in respiratory, nerve, blood systems and other organs, with poor prognosis, and repeated conditions during hospitalization, the parents abandoned treatment, and eventually the child died.

The proband's younger sister II-2, after birth also has a history of repeated hyperbilirubinemia, 8 days after birth due to hyperbilirubinemia admitted into external hospital where the baby received improvement in homocysteine detection (31.5 µmol/L). Due to 6 days after birth, the LC-MS/MS primary screening for detection presented abnormalities, the baby received a recall review and was diagnosed as MMA combined with homocystinuria (cblC type). After receiving intramuscular injection of vitamin B12, oral administration of L-carnitine tablets and betaine hydrochloride for treatment, the condition was stable and the baby received regular follow-up.

**Research methods**

**LC-MS/MS for detection**

The heel blood was collected from newborn 3 - 7 days after birth (because the proband vomited after birth, blood collection was delayed), the blood was dripped on the special hemofiltration paper for collection to form a blood spot with diameter over 8 mm, uniformly penetrating both sides. After air drying, the dry hemofiltration paper delivered to the neonatal disease screening center through a special cold chain. After being extracted via organic solvents containing amino acids and carnitine spectrum before and after treatment (see Table 1, 2). Analysis of indicators like plasma amino acids and acylcarnitine before and after treatment via LC-MS/MS.

**GS-MS for detection**

Those who tested positive in the primary screening were recalled for review. If they still tested positive in review, they would be recalled again by the neonatal disease screening center to receive GS-MS for detection of urine. The urine of child patient with MMA was collected, treated with urease, hydroxylammonium chloride, sodium hydroxide and hydrochloric acid, urea and protein were removed, 17-alkanoic acid was added as the internal standard and was extracted 2 times with ethyl acetate, and then methyl silane was derived with a mixture of 2-trifluoroacetamidine (trimethyl silane) and trimethylsilyl chloride. QP2010 GS-MS (Shimadzu Corporation, Japan) was applied to analyze methylmalonic acid level in urine (normal reference values: 0.2 - 3.6). As a semi-quantitative method, the finding was the ratio of the ion strength of methylmalonic acid to that of the inner standard 17-alkanic acid.

**Genetic testing**

A total of 5 mL of peripheral blood was extracted from the vein of those testing positive, EDTA anticoagulant was added, and sent to Hangzhou Chenyuan Medical Examination Laboratory for genetic testing. In total, 2 mL of peripheral blood was used to extract genomic DNA via Qiagen Blood DNA mini (Qiagen) kits, Qubit (Qubit® dsDNA HS Assay Kit, Invitrogen) was applied to determine the concentration, and stored at -20°C for reserve. Using the basic capture kit for detecting the gene of inherited metabolic diseases customized by Hangzhou Chenyuan Medical Examination Laboratory Co., Ltd., the library was constructed through multiprobe hybridization to target the sequence of areas rich in targets. The sequencing reaction was carried out via HiSeq 2500 high-flux sequencer, using BWA, GATK, Annovar and other software as well as database for analysis. In combination with the clinical phenotype of the sample, the mutation was interpreted pathogenically based on the latest ACMG standards and guidelines, the site and family were verified via Sanger sequencing for the mutations found.

**RESULTS**

**Clinical characteristics**

In total, 5 second-generation members of the family included 2 males and 3 females. The proband had onset at age 2 months, mainly manifested in repeated hyperbilirubinemia, breathing difficulties, severe hydrocephalus, pancytopenia, multi-organ functional damage. The proband’s younger sister after birth had repeated history of hyperbilirubinemia, with no progress in treatment after confirming diagnosis via LC-MS/MS. Analysis of indicators like plasma amino acids and acylcarnitine spectrum before and after treatment (see Table 1, 2).

**Molecular genetic analysis of members of the family**

The family showed autosomal recessive inheritance, and mutations in MMACHC were detected in all members. Both the proband and younger sister had complex heterozygous mutation in MMACHC, including c.609-G>A (p.W203X) nonsense mutation and c.467G>A (p.G156D) missense mutation. Based on the further genetic testing of MMACHC in their parents and older sis-
Table 1. Biochemical test results of proband before and after treatment.

<table>
<thead>
<tr>
<th>Age</th>
<th>C3 (0.28 - 4)</th>
<th>C3/C2 (0.02 - 0.19)</th>
<th>C3/C0 (0.01 - 0.16)</th>
<th>Homocysteine</th>
<th>Methylmalonic acid in urine (0.2 - 3.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 days (primary screening)</td>
<td>3.68</td>
<td>0.83</td>
<td>0.37</td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td>1 month and 16 days (review)</td>
<td>4.17</td>
<td>1.07</td>
<td>0.24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 months and 22 days</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>177.6</td>
<td>10.8</td>
</tr>
<tr>
<td>3 months and 1 day (after treatment)</td>
<td>3.45</td>
<td>0.37</td>
<td>0.22</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Biochemical test results of younger sister before and after treatment.

<table>
<thead>
<tr>
<th>Age</th>
<th>C3 (0.28 - 4)</th>
<th>C3/C2 (0.02 - 0.19)</th>
<th>C3/C0 (0.01 - 0.16)</th>
<th>Homocysteine</th>
<th>Methylmalonic acid in urine (0.2 - 3.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 days (primary screening)</td>
<td>11.09</td>
<td>0.86</td>
<td>0.71</td>
<td>31.5</td>
<td>-</td>
</tr>
<tr>
<td>17 days (review)</td>
<td>8.74</td>
<td>1.61</td>
<td>0.59</td>
<td>13.3</td>
<td>328.2</td>
</tr>
<tr>
<td>24 days (after treatment)</td>
<td>1.55</td>
<td>0.11</td>
<td>0.03</td>
<td>14.4</td>
<td>4.62</td>
</tr>
</tbody>
</table>

Table 3. Gene mutation for families.

<table>
<thead>
<tr>
<th>Patient</th>
<th>gene</th>
<th>exon/ introne</th>
<th>Chromosome positions</th>
<th>Transcript number</th>
<th>Mutant sites</th>
<th>Disease types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband</td>
<td>MMACHC</td>
<td>exon4</td>
<td>chr1: 45974647/ chr1: 45974505</td>
<td>NM_015506.2</td>
<td>c.609G&gt;A (p.W203X)/ c.467G&gt;A (p.G156D)</td>
<td>compound heterozygous mutation</td>
</tr>
<tr>
<td>Younger sister</td>
<td>MMACHC</td>
<td>exon4</td>
<td>chr1: 45974647/ chr1: 45974505</td>
<td>NM_015506.2</td>
<td>c.609G&gt;A (p.W203X)/ c.467G&gt;A (p.G156D)</td>
<td>compound heterozygous mutation</td>
</tr>
<tr>
<td>Older sister</td>
<td>MMACHC</td>
<td>exon4</td>
<td>chr1: 45974647/ chr1: 45974505</td>
<td>NM_015506.2</td>
<td>c.609G&gt;A (p.W203X)/ c.467G&gt;A (p.G156D)</td>
<td>carrier (normal phenotype)</td>
</tr>
<tr>
<td>Father</td>
<td>MMACHC</td>
<td>exon4</td>
<td>chr1: 45974647/ chr1: 45974505</td>
<td>NM_015506.2</td>
<td>c.609G&gt;A (p.W203X)/ c.467G&gt;A (p.G156D)</td>
<td>carrier (normal phenotype)</td>
</tr>
<tr>
<td>Mother</td>
<td>MMACHC</td>
<td>exon4</td>
<td>chr1: 45974647/ chr1: 45974505</td>
<td>NM_015506.2</td>
<td>c.609G&gt;A (p.W203X)/ c.467G&gt;A (p.G156D)</td>
<td>carrier (normal phenotype)</td>
</tr>
</tbody>
</table>

Bioinformatics analysis on c.467G>A (p.G156D) gene mutation
After querying databases such as dbSNP, ExAC, genomAD\1000 genome project, our team did not find the possibility that c.467G>A (p.G156D) had polymorphism in MMACHC. Polyphen-2 software had a prediction score of 0.906 for this mutation, indicating "potentially harmful" and SIFT software for 0, indicating "harmful". The MutationTaster software predicted a score of 0.99999999999911317, indicating "pathogen-
ic". Comparing the sequences of protein amino acids such as humans, chimpanzees, monkeys, whales, chickens, wireworms, and yeasts, it was found that the related site had important biological functions because the sequences were highly conservative among species. DUET software predicted that p.G156D mutation could disrupt the stability of the protein in MMACHC (mC-SM, xenon G, -1.822 Kcal/mol), thus affecting the normal configuration of the protein and, in turn, its normal activity. The bioinformatics analysis on c.467G>A (p.G156D) mutation was shown in Figure 2.

DISCUSSION
MMA prevalence varies greatly from country to country, which is reported as significantly higher domestically than abroad, with 7.8 million newborns in China showing a prevalence rate of about 1:15,000 and MMA prevalence in Shandong Province of 1:3,920 [10]. Newborns and adults are likely to be affected with MMA. The prognosis of patients with MMAcbIC is related to the age of onset. The earlier the onset, the heavier the symptoms and the worse the prognosis [11]. Because MMA is an intermediate product in the metabolic pathways of isoleucine, valine, methanesulfonic acid and other amino acids and odd chain fatty acids, it is normally converted into amber acid under the action of MCM and its Cbl-C and participates in the tricarboxylic acid cycle (TCA cycle). Once there are mutase defects or cobalamin metabolic disorders, this will lead to a large number of metabolites such as methylmalonic and propanoic acid accumulating in the body, resulting in functional damage to nerve, blood, kidneys, liver, and other organs [12-14]. The LC-MS/MS technology is available to detect various amino acids and acyl-carnitine at the same time through a drop of blood, screening for various genetic metabolic diseases (including amino acid and organic acid metabolism diseases, urea circulation disorder, fatty acid oxidation disorder), with the advantages of screening various diseases through a single experiment. The technology has been in place since January 2015 at the Zaozhuang Branch Center of the Newborn Disease Screening Center in Shandong Province with a view to early diagnosis and good intervention. MMA clinical diagnosis and typing are as follows: Those who tested positive in the primary screening were recalled for a review. If still tested positive in the review, they would be recalled again by the neonatal disease screening center to receive GS-MS for detection of urine, gene mutation analysis as well as conventional laboratory test for clear diagnosis. Diagnosis basis: (1) feeding difficulties, prolonged jaundice, repeated vomiting, drowsiness, convulsions, poor muscular tension, underdevelopment and other clinical manifestations; abnormalities in general biochemical examination, including metabolic acidosis, liver failure, and anemia. (2) LC-MS/MS detection findings revealed that increased blood C3 and C3/C2, sometimes with C0 or reduced methionine. (3) GS-MS resulted in elevated methyl-
Figure 2. Biochemical information analysis of c.467G>A (p.G156D) mutation.

Note: A: Polyphen-2 for prediction; B: SIFT for prediction; C: MutationTaster for prediction; D: DUET for prediction.
malaric acid in urine and/or methyl citric acid. (4) Excluding secondary MMA due to nutritional factors such as folic acid and vitamin B12 deficiency. (5) Analyzing the relevant gene mutations to identify disease-causing mutation sites [15,16]. According to the test findings of child patient’s homocysteine (normal reference value: 4.44 - 13.56 μmol/L) for typing, the normal with isolated MMA, the elevated with MMA combined with homocystinuria (combined MMA for short). Based on vitamin B12 load testing for typing, that is, daily muscular injection of 1 mg vitamin B12 for 3 to 5 days. If the clinical symptoms improve, blood C3, C3/C2, and methylmalonic acid levels in urine decreased by 50% in comparison with those before treatment, which is B12 effective type, otherwise ineffective. Based on the age of onset division into early onset (age of onset ≤ 1 year) and late onset (age of onset > 1 year).

At present, 26 genes related to MMA morbidity have been found, and common pathogenetic genes are MUT, MMIAA, MMIB, MMACHC, MMADHC, LMBRD1 etc. Liu Yi et al. have shown that in China combined MMA accounts for about 70% of cases, which is the main biochemical type, whereas the cblC defect (MMAcblC) caused by MMACHC defect is the main cause of combined MMA in China, and MMAcblC shows autosomal recessive inheritance. MMA children present complex clinical manifestations, which is difficult to identify, and needs to rely on biochemical and genetic diagnosis. In MMACHC, cblC coding gene is located in the euchromosome 1p34.1, containing 5 exons, genes about 10.7 kb in length; the exons 1 to 4 are the coding areas, in length 849 bp, 282 amino acids consisting of cblC protein via encoding and exon 5 is a non-coding area [17]. MMACHC mutation results in functional defects of cblC protein, more than 50 kinds of mutations have been found, most of which are located on exons 3 and 4; genotypes, clinical phenotypes as well as ethnology have a certain correlation [18]. In this work, the proband had combined MMA with early onset, because in the early LC-MS/MS for detection of children there was no obvious increase. It is recommended to receive further genetic testing, but the parents refused it. Children thereby did not get early diagnosis, and the disease progressed. Even if later, they had the gene testing to confirm diagnosis and received treatment, the child had had serious complications, the child's parents abandoned treatment and finally the child died. Parents of children patients refused to improve family certification and their re-pregnancy was not given prenatal diagnosis. The third birth, the proband’s younger sister tested positive in initial screening. After receiving GS-MS detection of urine, gene mutation analysis, and conventional laboratory examination, the baby was clearly diagnosed as combined MMA based on diagnostic standards. Early interventions did not result in serious complications, with continuous follow-up. In this work, the proband and his younger sister were diagnosed with combined MMA, and the MMA disease-causing gene was quickly screened through Ion Torrent semiconductor sequencing technology. Our team detected c.609G>A and c.467G>A heterozygous mutations in MMAC in both, which were inherited from the father and mother, respectively, and called compound heterozygous mutation. The c.609G>A (p.W203X), the pathogenic hotspot mutation in MMACHC, is a common mutation site in children with cblC disease in China and the nonsense mutation, in whose protein the 203rd amino acid is changed from tryptophan to a stop codon (p.W203*). In the protein of c.467G>A the 156th amino acid was changed from glycine to aspartic acid (p.G156D) which was a missense mutation, and this type mutation site has not been reported in the domestic literature but in foreign literature it has. It only found that a patient with MMA combined with homocystinuria carried this mutation [19]. In accordance with ACMG guidelines, the mutation features clinical significance unfounded. Parents and older sister carrying these two heterozygous mutations did not have onset but the proband and younger sister had, suggesting that maybe the combined effect of heterozygous mutation from parents caused the onset. In the domestic literature and Zaozhuang, the c.467G>A (p.G156D) mutation has not been found, indicating that c.467G>A (p.G156D) may not be a MMA mutation hotspot in China. The findings expand and enrich mutation types in MMACHC, provide a risk assessment for the fertility of family members, screen normal fetuses via prenatal diagnostic techniques, and prevent the birth of defective children.

Declaration of Interest:
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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