Original Article

DOI: 10.5582/irdr.2023.01016

A regionally adapted HRM-based technique to screen MMACHC carriers for methylmalonic acidemia with homocystinuria in Shandong Province, China

Haining Yang[1,2,3], Mian Li[1,2,3], Liang Zou[4], Hui Zou[5], Yan Zhao[1,2,3,*], Yazhou Cui[1,2,3,*], Jinxiang Han[1,2,3,*]

[1] Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;
[2] NHC Key Laboratory of Biotechnology Drugs (Shandong Academy of Medical Sciences), Ji'nan, Shandong, China;
[3] Key Lab for Rare & Uncommon Diseases of Shandong Province, Ji'nan, Shandong, China;
[4] Bone Biomechanics Engineering Laboratory of Shandong Province, Neck-Shoulder and Lumbocrural Pain Hospital of Shandong First Medical University, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan, China;
[5] Ji'nan Maternity and Child Care Hospital, Ji'nan 250000, Shandong, China.

SUMMARY Methylmalonic acidemia with homocystinuria (MMA-cblC) is an autosomal recessive genetic disorder of organic acid metabolism. Shandong, a northern province of China, has a significantly high incidence of about 1/4,000, suggesting a high carrying rate among the local population. The current study established a PCR technique involving high-resolution melting (HRM) to screen for carriers based on hotspot mutation analysis to further develop a preventive strategy to reduce the local incidence of this rare disease. Whole-exome sequencing of 22 families with MMA-cblC and a comprehensive literature review were used to identify MMACHC hotspot mutations in Shandong Province. Subsequently, a PCR-HRM assay based on the selected mutations was established and optimized for large-scale hotspot mutation screening. The accuracy and efficiency of the screening technique was validated using samples from 69 individuals with MMA-cblC and 1,000 healthy volunteers. Six hotspot mutations in the MMACHC gene (c.609G>A, c.658_660delAAG, c.80A>G, c.217C>T, c.567dupT and c.482G>A), which account for 74% of the alleles associated with MMA-cblC, were used to establish a screening technique. The established PCR-HRM assay detected 88 MMACHC mutation alleles in a validation study with 100% accuracy. In the general population in Shandong, the carrying rate of 6 MMACHC hotspot mutations was 3.4%. In conclusion, the 6 hotspots identified cover the majority of the MMACHC mutation spectrum, and the Shandong population has a particularly high carrying rate of MMACHC mutations. The PCR-HRM assay is highly accurate, cost-effective, and easy to use, making it an ideal choice for mass carrier screening.

Keywords carrier screening, methylmalonic aciduria, MMACHC, PCR-HRM, mutation

1. Introduction

Methylmalonic acidemia (MMA) is a severe autosomal recessive inborn error of organic acid metabolism. The estimated incidence of MMA ranges from 1/48,000 to 1/250,000 in different countries worldwide (*1*). Its incidence is particularly high, 1/4,000, in the population of Shandong, a northern province of China (*2*). MMA-cblC is MMA combined with homocystinuria caused by defects in cobalamin biosynthesis, and it is the most common form of MMA in China, accounting for approximately 70% of all MMA cases (*3-4*). Age of onset ranges from shortly after birth to late adulthood, with clinically heterogeneous manifestations that include vital organ damage, recurrent vomiting, mental retardation, and progressive developmental delay; some severe cases even lead to death (*5*).

However, two factors make MMA-cblC one of the few rare diseases with a better prognosis for most patients. First, due to the increasing prevalence of newborn screening based on tandem mass spectrometry (MS/MS), more infants are being diagnosed and treated early. Second, early supplementation with vitamin B12 and L-carnitine can have a significant therapeutic benefit, reversing some clinical phenotypes (*6*). However, a screening strategy to identify mutation carriers to

reduce prenatal incidence has not yet been explored. The apparent prevalence of MMA-cblC in Shandong Province suggests a significantly high carrying rate of MMA gene mutations in the general population. Therefore, the current study investigated hotspot mutations of the MMACHC gene and their prevalence in MMA-cblC, and it evaluated whether carrier screening is suitable for primary prevention of that condition in Shandong. This study also developed a cost-effective strategy that could be used in a large population.

2. Materials and Methods

2.1. Clinical samples

MMA-cblC was diagnosed according to previously reported criteria (*7-8*). Twenty-two patients diagnosed with MMA-cblC from January 2017 to January 2019 at the Jinan Maternity and Child Care Hospital (Shandong, China) and their parents were included in the spot mutation analysis using peripheral blood samples. Dry blood spots were obtained from 69 infants with MMA-cblC and one of the six hotspot mutations from January 2019 to January 2022 in Shandong, and those blood spots were used to develop and validate a PCR-HRM assay. Peripheral blood samples from 1,000 healthy volunteers in Shandong province were obtained between January 2019 to December 2021 as part of a study to screen for MMACHC mutation carriers. All the samples were collected with informed consent of the individual or his/her parent/guardian. This study was approved by the Institutional Ethics Committee of the Shandong Medicinal Biotechnology Centre

2.2. Whole exome sequencing and analysis

Genomic DNA was extracted from peripheral blood from 22 MMA-cblC pedigrees using the Vazyme Blood DNA Kit (Vazyme, China). A sequencing library was then constructed using the AmpliSeq™exome kit (Thermo Fisher Scientific, USA). Ion torrent S5 (Thermo Fisher Scientific, USA) was used to perform sequencing according to the manufacturer's instructions. FASTQ files were automatically generated from the sequenced original image data by base calling. Read pairs were concatenated and filtered to remove low-quality bases. Read alignments were performed against the hg19 reference genome. Variant calling and annotation was performed using the CLC Genomics Workbench (Qiagen, Germany) (*9*).

2.3. Establishment and optimization of the PCR-HRM assay

PCR-HRM primers were designed using the software Primer Express v3.0 in accordance with the reference sequence of the MMACHC gene (NG_013378.2) in NCBI. PCR primers specific for each hotspot mutation were designed to yield 100-150-bp products with a Tm between 58-60℃.

PCR-HRM was performed on a LightCycler 480 real-time PCR system (Roche Diagnostics, Germany). The total volume of the PCR reaction was 20 μL, including 10 μL of Roche Master Dye premix (2×), 1 μL of forward and 1 μL of reverse primers (4 μM) and 25 ng of the genomic DNA template. The reaction conditions were as follows: initial denaturation at 95℃ for 5 min, 45 cycles of denaturation at 95℃ for 10 s, annealing at 55℃ for 15 s, and extension at 72℃ for 10 s with fluorescence reading and single point acquisition mode. After amplification, melting curve analysis was performed in three steps: denaturation at 95℃ for 1 min, renaturation at 40℃ for 1 min, followed by continuous fluorescence reading mode at 65-95℃ with a rise rate of 0.02℃/s and data acquisition of 25 times/℃. Raw data were analyzed and normalized using the software supplied with the PCR machine. Negative controls were used as a reference curve to generate difference plots. The normalized melting curve and difference plots were then analyzed for mutation genotype.

2.4. Sanger sequencing

Sanger sequencing was used to validate the accuracy of HRM on an ABI-3730XL Genetic Analyser (Thermo Fisher Scientific, USA). The results were analyzed using the software Chromas.

3. Results

The flowchart for this study is shown in Figure 1. Hotspot mutations were first evaluated in 22 patients with MMA-cblC and their parents using whole exome sequencing. MMACHC mutations were found in both alleles in all patients (Table 1). Of them, 16 children were carriers of compound heterozygous variants and the remaining 6 children carried compound homozygous variants. In total, 9 known pathogenic variants in the MMACHC gene were identified. The most frequent mutation was c.609 G>A, which was detected in half of the patients (50.0%, 11/22). The second most common mutation, c.658_660delAAG, was detected in 10 (45.45%, 10/22). More than half of the pathogenic mutations were classified as nonsense and frameshift mutations.

The hotspot mutation list was then compared to two previous studies of the MMACHC mutation spectrum in Shandong Province (*1,10*). The top hotspot mutations in all three studies were c.609G>A, c.658_660delAAG, c.80A>G, c.217C>T, c.567dupT, and c.482G>A, representing 75.94% of MMACHC mutation sites. Therefore, these top hotspot mutations could be used as an ideal choice for massive carrier screening.

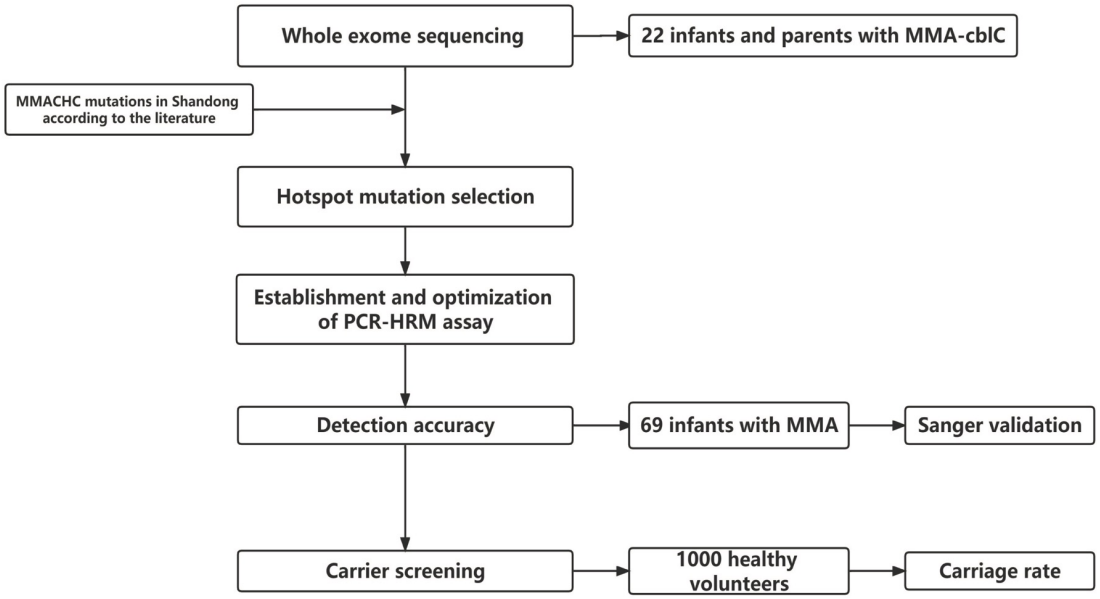

Figure 1. Flowchart for this study.

Table 1. Detailed information on the 22 MMA pedigrees for exome sequencing in this study

No.	Gene	Nucleotide changes	Amino acid changes	Mutation type	Source of variation	Homo./Het.
1	MMACHC	c.80A>G	p.Gln27Arg	Missense	Father	Het.
		c.609G>A	p.Trp203Ter	Nonsense	Mother	
2	MMACHC	c.658_660delAAG	p.Lys220del	Deletion	Parents	Homo.
3	MMACHC	c.217C>T	p.Arg73Ter	Nonsense	Father	Het.
		c.658-660delAAG	p.Lys220del	Deletion	Mother	
4	MMACHC	c.658-660delAAG	p.Lys220del	Deletion	Parents	Homo.
5	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Father	Het.
		c.622_627delTG	p.Val209fs	Frame shift	Mother	
6	MMACHC	c.445_446delTG	p.Cys149fs	Frame shift	Father	Het.
		c.615C>A	p.Tyr205Ter	Nonsense	Mother	
7	MMACHC	c.482G>A	p.Arg161Gln	Missense	Father	Het.
		c.567dupT	p.Ile190fs	Frame shift	Mother	
8	MMACHC	c.482G>A	p.Arg161Gln	Missense	Parents	Homo.
9	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Parents	Homo.
10	MMACHC	c.658_660delAAG	p.Lys220del	Deletion	Parents	Homo.
11	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Father	Homo.
12	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	-	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Mother	
13	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	-	Het.
		c.658_660delAAG	p.Lys220del	Deletion	-	
14	MMACHC	c.445_446delTG	p.Cys149fs	Frame shift	Father	Het.
		c.609G>A	p.Trp203Ter	Nonsense	Mother	
15	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Father	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Mother	
16	MMACHC	c.80A>G	p.Gln27Arg	Missense	Father	Het.
		c.609G>A	p.Trp203Ter	Nonsense	Mother	
17	MMACHC	c.217C>T	p.Arg73Ter	Nonsense	Mother	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Father	
18	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Mother	Het.
		c.626_627delTG	p.Val209fs	Frame shift	-	
19	MMACHC	c.445_446delTG	p.Cys149fs	Frame shift	Mother	Het.
		c.615C > A	p.Tyr205Ter	Nonsense	Father	
20	MMACHC	c.482G>A	p.Arg161Gln	Missense	Mother	Het.
		c.567dupT	p.Ile190fs	Frame shift	Father	
21	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Mother	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Father	
22	MMACHC	c.481C>T	p.Arg161Ter	Nonsense	Mother	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Father	

Specific primers for the six hotspot mutations were then designed (Table 2), and a rapid PCR-HRM technique was established to detect mutations in 6 cases of MMA-cblC (Table 3). As shown in Figure 2, the melting peak and melting curve of each hotspot mutation site differed significantly different from the others, and those of the mutant and wild genotypes of each site were clearly distinct. The established PCR-HRM assay is fast, completed in half an hour, and data are easily analyzed using the software provided with the real-time PCR system.

To evaluate the accuracy of PCR-HRM techniques, PCR-HRM assays were performed on samples from 69 infants confirmed to have MMA-cblC and at least one of the six hotspot mutations. A total of 88 alleles of six selected MMACHC hotspot mutations were found. The PCR-HRM results were in 100% agreement with the Sanger sequencing of the complete exons (Table 4).

Finally, pilot screening for carriers of MMACHC hotspot mutations was performed on 1,000 healthy volunteers, and 34 MMACHC hotspot mutations were identified (Table 5). Therefore, results revealed a carrying rate of 3.4% for MMACHC in the Shandong population.

Table 3. PCR-HRM primers for *MMACHC* hotspot mutation sites

Mutation site	Name	Sequence (5′-3′)
c.80A>G	MMACHC-1F	CAGAGCTGAAGCAGAAGATCGA
	MMACHC-1R	CCCTAGAACAGCAGGAGGGATA
c.217C>T	MMACHC-2F	CACGCCTGCCATGTTTGAC
	MMACHC-2R	CCAGATGGTAGGCCACACACT
c.567dupT	MMACHC-3F	CCAGGGATAGAGGTGCCAGAT
	MMACHC-3R	TCACGCCAGTGGAAATTGAAG
c.482G>A	MMACHC-4F	CCAGCGCATATCAGGTGTGT
	MMACHC-4R	ATCCCTGGCAGCAGCACTAC
c.658_660	MMACHC-5F	CCTACTCGAAGGCTTCAATTTCC
delAAG	MMACHC-5R	CCGGGGAGGGAGAACTAGG
c.609G>A	MMACHC-6F	AGCTGACCGTATCGCCCTACT
	MMACHC-6R	AGTAGGCCTTCTGCTCTTCTGAGT

Table 2. Statistics on pathogenic *MMACHC* mutation sites according to the results of exosome sequencing and a literature review

Mutation site	This study ($N = 44$)		Li Y [10] ($N = 128$)		Wang F [1] ($N = 94$)	
	Allele number	Allele frequency	Allele number	Allele frequency	Allele number	Allele frequency
c.609G>A	13	29.55%	43	33.59%	52	55.43%
c.658_660delAAG	13	29.55%	13	10.16%	12	13.04%
c.567dupT	2	4.55%	6	4.69%	2	2.17%
c.80A>G	2	4.55%	15	11.72%	1	1.09%
c.217C>T	2	4.55%	1	0.78%	1	1.09%
c.482G>A	4	9.10%	19	14.84%	1	1.09%
Total	36	81.85%	97	75.99%	69	73.91%

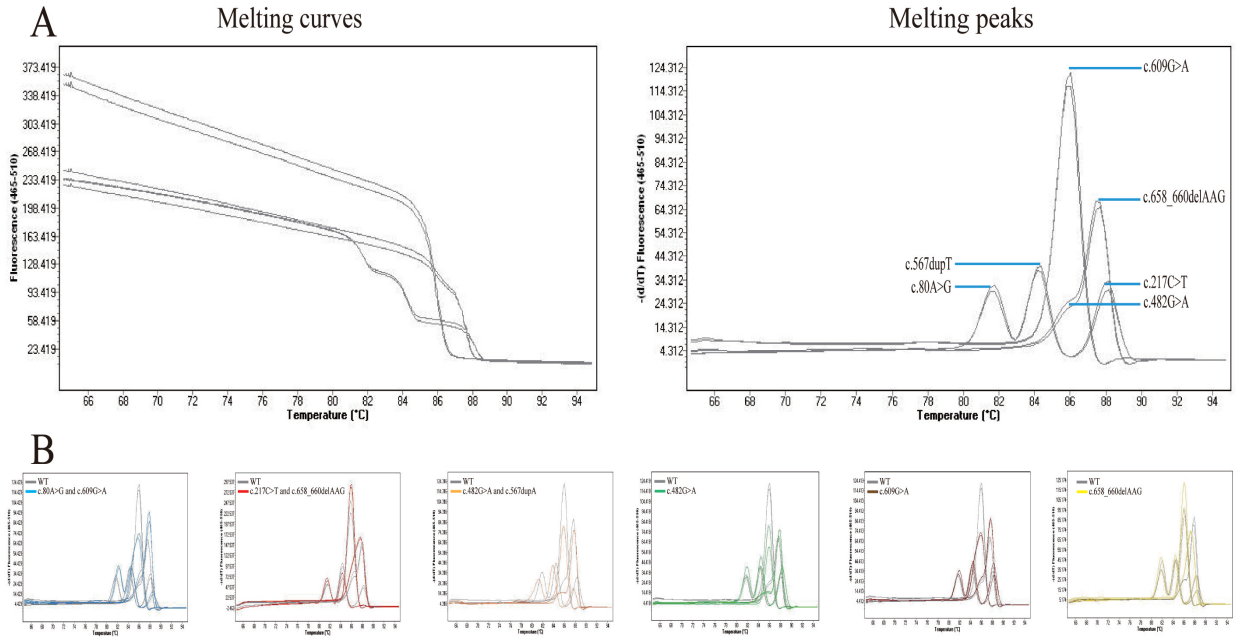

Figure 2. Performance of the established PCR-HRM technique in detecting mutations of the *MMACHC* gene. (A) The melting curves and peaks of hotspot mutation sites. **(B)** The melting peaks of six hotspot mutations in patients with MMA-cblC.

Table 4. Analysis of *MMACHC* mutation sites in 69 infants with MMA

Mutation site	Number (%)	Allele frequency (%)
c.80A>G	12	13.64
c.217C>T	1	1.14
c.567dupT	10	11.36
c.482G>A	11	12.50
c.658_660delAAG	19	21.59
c.609G>A	35	39.77
Total alleles	88	100

Table 5. Analysis of *MMACHC* mutation sites in 1000 healthy volunteers

Mutation site	Number	Carriage rate (%)	Allele frequency (%)
c.80A>G	3	0.3	8.82
c.217C>T	3	0.3	8.82
c.567dupT	1	0.1	2.94
c.482G>A	5	0.5	14.7
c.658_660delAAG	10	1	29.41
c.609G>A	12	1.2	35.29
Total alleles	34	3.4	100

4. Discussion

In autosomal recessive or X-linked recessive inheritance, all carriers have compound heterozygous pathogenic variants but do not exhibit any phenotypic characteristics. The aim of carrier screening is to identify asymptomatic individuals carrying the causative variant at an early stage so that appropriate fertility counselling can be provided. Tay-Sachs disease was the first genetic disease for which population-based carrier screening was implemented, and research has provided clear evidence that such efforts can dramatically reduce its incidence (*11-14*).

The causative gene for MMA-cblC was first identified as MMACHC by Lerner-Ellis *et al*. in 2006. According to the Human Gene Mutation Database (HGMD), 127 MMACHC mutation sites have been reported (*15*). MMACHC mutations vary by country and region. For example, c271dupA accounts for about 50% of MMACHC mutations in European populations, while c.331C>T is common in French-Canadian mixed-race populations and c.394C>T in Middle Eastern populations (*16,17*). In 2010, Liu *et al*. analyzed mutations of the MMACHC gene in children with MMA-cblC in China (*18*). The high incidence of the c.80A>G, C.609G>A and 658_660delAAG mutations, which account for more than 50% of the MMA alleles, may be related to the early founder effect of the Chinese population. The c.217C>T and c.482G>A mutations are also common in groups of Chinese patients with MMA-cblC, which may be related to the CpG island in the human genome (*19*). Although Shandong has a significantly higher incidence of MMA compared to other regions, the current analysis revealed that the spectrum of MMACHC mutations in Shandong is consistent with previous findings in the Chinese population. In light of the assay's substantial coverage of hot spot mutations, using a PCR-based screening technique with low throughput and a low cost is feasible.

This study found an MMACHC carriage rate of 34 per 1,000 in the Shandong population, similar to the finding in an earlier study (210/6800) (*10*). Compared to existing carrier screening techniques, the PCR-HRM technique established here is suitable for large-scale screening of pathogenic MMACHC gene carriers to reduce the incidence of MMA-cblC in Shandong Province because of its high efficiency, accuracy, low cost, short time, and limited workload in analyzing results. However, widespread promotion of this strategy for carrier screening still faces many challenges, including public awareness and education, informed consent, interpretation of results, and genetic counselling.

Acknowledgements

The authors would like to sincerely thank the patients and volunteers who took part in this study.

Funding: This work was partially funded by the Academic Promotion Program of Shandong First Medical University (2019LJ001) and the Key Research and Development Project of Shandong Province (2021ZDSYS27).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Wang F, Han L, Yang Y, Gu X, Ye J, Qiu W, Zhang H, Zhang Y, Gao X, Wang Y. Clinical, biochemical, and molecular analysis of combined methylmalonic acidemia and hyperhomocysteinemia (cblC type) in China. J Inherit Metab Dis. 2010; 33 Suppl 3:S435-442.
2. Zhou X, Cui Y, Han J. Methylmalonic acidemia: Current status and research priorities. Intractable Rare Dis Res. 2018; 7:73-78.
3. Liu Y, Li X, Wang Q, Ding Y, Song J, Yang Y. Five novel SUCLG1 mutations in three Chinese patients with succinate-CoA ligase deficiency noticed by mild methylmalonic aciduria. Brain Dev. 2016; 38:61-67.
4. Deme JC, Hancock MA, Xia X, Shintre CA, Plesa M, Kim JC, Carpenter EP, Rosenblatt DS, Coulton JW. Purification and interaction analyses of two human lysosomal vitamin B12 transporters: LMBD1 and ABCD4. Mol Membr Biol. 2014; 31:250-261.
5. Fischer S, Huemer M, Baumgartner M, *et al*. Clinical presentation and outcome in a series of 88 patients with the cblC defect. J Inherit Metab Dis. 2014; 37:831-840.
6. Wang C, Li D, Cai F, Zhang X, Xu X, Liu X, Zhang C, Wang D, Liu X, Lin S, Zhang Y, Shu J. Mutation spectrum of MMACHC in Chinese pediatric patients with cobalamin C disease: A case series and literature review. Eur J Med Genet. 2019; 62:103713.
7. Liu Y, Liu YP, Zhang Y, *et al.* Heterogeneous phenotypes, genotypes, treatment and prevention of 1 003 patients

with methylmalonic acidemia in the mainland of China. Zhonghua Er Ke Za Zhi. 2018; 56:414-420. (in Chinese w/ English abstract)
8. Forny P, Hörster F, Ballhausen D, *et al.* Guidelines for the diagnosis and management of methylmalonic acidaemia and propionic acidaemia: First revision. J Inherit Metab Dis, 2021; 44:566-592.
9. Liu CH, Di YP. Analysis of RNA sequencing data using CLC Genomics Workbench. Methods Mol Biol. 2020; 2102:61-113.
10. Li Y, Sun M, Li P, Tian L, Guo Y, Li G, Li R, Yan Y, Li Q, Zou H. Analysis of biochemical and genetic screening results for neonatal methylmalonic acidemia. Zhonghua Er Ke Za Zhi, 2023; 38:54-59. (in Chinese w/ English abstract)
11. Edwards JG, Feldman G, Goldberg J, Gregg AR, Norton ME, Rose NC, Schneider A, Stoll K, Wapner R, Watson MS. Expanded carrier screening in reproductive medicine-points to consider: A joint statement of the American College of Medical Genetics and Genomics, American College of Obstetricians and Gynecologists, National Society of Genetic Counselors, Perinatal Quality Foundation, and Society for Maternal-Fetal Medicine. Obstet Gynecol. 2015; 125: 653-662.
12. CD Genetics. Committee opinion No. 690: Carrier Screening in the Age of Genomic Medicine. Obstet Gynecol. 2017; 129: e35-e40.
13. Martin J, Asan, Yi Y, Alberola T, Rodríguez-Iglesias B, Jiménez-Almazán J, Li Q, Du H, Alama P, Ruiz A, Bosch E, Garrido N, Simon C. Comprehensive carrier genetic test using next-generation deoxyribonucleic acid sequencing in infertile couples wishing to conceive through assisted reproductive technology. Fertil Steril. 2015; 104:1286-1293.
14. Wapner RJ, Biggio, Jr. JR. Commentary: Expanded carrier screening: How much is too much? Genet Med. 2019; 21:1927-1930.
15. Liu F, Wu Y, Li Z, Wan R. Identification of MMACHC and ZEB2 mutations causing coexistent cobalamin C disease and Mowat-Wilson syndrome in a 2-year-old girl. Clin Chim Acta. 2022; 533:31-39.
16. Morel CF, Lerner-Ellis JP, Rosenblatt DS. Combined methylmalonic aciduria and homocystinuria (cblC): Phenotype-genotype correlations and ethnic-specific observations. Mol Genet Metab. 2006; 88:315-321.
17. Nogueira C, Aiello C, Cerone R, *et al*. Spectrum of MMACHC mutations in Italian and Portuguese patients with combined methylmalonic aciduria and homocystinuria, cblC type. Mol Genet Metab. 2008; 93:475-480.
18. Liu MY, Yang YL, Chang YC, Chiang SH, Lin SP, Han LS, Qi Y, Hsiao KJ, Liu TT. Mutation spectrum of MMACHC in Chinese patients with combined methylmalonic aciduria and homocystinuria. J Hum Genet. 2010; 55:621-626.
19. Cooper DN, Youssoufian H. The CpG dinucleotide and human genetic disease. Hum Genet. 1988; 78:151-155.

Received February 10, 2023; Revised February 21, 2023; Accepted February 24, 2023.

**Address correspondence to:*
Jinxiang Han, Yazhou Cui, and Yan Zhao, Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences; NHC Key Laboratory of Biotechnology Drugs (Shandong Academy of Medical Sciences); Key Lab for Rare & Uncommon Diseases of Shandong Province, 6699 Qingdao Road, Ji'nan 250117, Shandong, China.
E-mail: jxhan9888@aliyun.com (JH); yzcui@sdfmu.edu.cn (YC); zhaoyan@sdfmu.edu.cn (YZ)

Released online in J-STAGE as advance publication February 26, 2023.