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RESEARCH ARTICLE

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A molecular-beacon-based asymmetric PCR assay for detecting polymorphisms related to folate metabolism

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Abstract

Background: Polymorphisms (rs1801133 or C677T; rs1801131 or A1298C) of the *MTHFR* gene and rs1801394 (A66G) of the *MTRR* gene are important genetic determinants of folate metabolism. A convenient, sensitive, and reliable method is required to detect polymorphisms for the precise supplementation of folate.

Methods: A rapid detection method based on molecular beacon probes that can detect rs1801133, rs1801131, and rs1801394 simultaneously was developed in this study. Specific primers and probes were designed, and the amplification system and conditions were optimized. We applied our method to a group of 500 unrelated women of gestational age in the Dongguan region of Guangdong Province in China. The clinical performance of this assay was evaluated by testing 94 samples in comparison with Sanger sequencing.

Results: The molecular-beacon-based PCR assay we established is extremely sensitive, with a detection limit of 2 ng/μL of genomic DNA, and validated by direct sequencing in a blind study with 100% concordance.

Conclusion: The results demonstrate that our molecular-beacon-based asymmetric PCR assay is an easy, reliable, high-yield, and cost-effective method for the simultaneous detection of three polymorphisms related to folate metabolism. It could help evaluate the risk of perinatal-neonatal neural tube malformation, pregnancy hypertension, and other diseases and guide the individualized supplementation of folic acid. Data on the spectrum of mutations in the Dongguan District in this study are beneficial for guiding the supplementation of folic acid.

KEYWORDS

folate metabolism, molecular-beacon, MTHFR, MTRR, polymorphism

Qi Peng and Shengbin Liao contributed equally to this work.

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1 | INTRODUCTION

Folate plays an important role in numerous metabolic processes, which can affect DNA synthesis and are involved in the methylation of DNA and protein.[1,2] Folate metabolism disorder can lead to hyperhomocysteinemia (Hcy), which is associated with an increased risk of many disorders, including birth defects, pregnancy complications, vascular and neurodegenerative diseases, diabetes, neuropsychiatric disorders, and cancers.[3,4] The major genetic factors are polymorphisms in genes encoding metabolizing enzymes, such as rs1801133 (C677T) and rs1801131 (A1298C) of methylenetetrahydrofolate reductase (MTHFR) or rs1801394 (A66G) of methionine synthase reductase (MTRR).

MTHFR catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is essential for folate-mediated one-carbon metabolism.[5,6] rs1801133, with a C to T transition at base pair 677 resulting in an alanine-to-valine substitution,[7] and rs1801131, with an A to C transition at base pair 1298 leading to a glutamate to alanine substitution, have been confirmed to decrease enzyme activity.[8] The reduced mean enzyme activity has been shown to be approximately 30% normal in homozygotes and approximately 65% normal in heterozygotes.[8-10]

MTRR catalyzes the reductive methylation of MTRs and regenerates functional MTRs during folate metabolism.[11] The MTRR restores methionine synthase (MTR) activity and is consequently a critical determinant of Hcy levels.[12] The activity of this enzyme may vary owing to different genetic variations. The most common polymorphism in the MTRR gene is the substitution of A for G at nucleotide 66 (rs1801394), which decreases the enzyme activity and the rate of Hcy remethylation.[13]

The utilization ability of folate can be evaluated at the molecular level through the detection of MTHFR and MTRR gene polymorphisms. Therefore, it is necessary to establish a high-throughput, low-cost, fast, and simple screening method for gene mutations of folate metabolism-related enzymes to achieve rapid clinical detection and large-scale screening. In this study, we developed a molecular-beacon-based PCR assay for the simultaneous detection of rs1801133 and rs1801131 of the MTHFR gene and rs1801394 of the MTRR gene, which can help to screen out high-risk individuals who are likely to have folate deficiency and realize individualized folate supplementation that provides the exact folate supplementation plan and quantity according to people's needs.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was conducted in accordance with the Declaration of Helsinki, and all procedures were approved by the Institutional Medical and Ethics Committee of Dongguan Eighth People's Hospital. All specimens and survey data were obtained with written informed consent from all participants prior to study.

2.2 | Subjects

Between May 2018 and September 2018, a total of 500 unrelated, apparently healthy women of Han nationality aged 20-40 years who came to our hospital for prepregnancy care were recruited in the study. After obtaining informed written consent, peripheral blood samples were drawn from all participants into EDTA anticoagulated tubes.

2.3 | Genomic DNA extraction

Genomic DNA was extracted from 300 μL of blood using the QIAamp Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The isolated DNA samples were stored at −20°C until further analysis. The quality and quantity of the isolated DNA were analyzed with a NanoDrop 2000 instrument (Thermo Fisher Scientific).

2.4 | Primers and molecular beacon probe design

Based on the *MTHFR* and *MTRR* genes in *Homo sapiens* published in NCBI, Oligo 7.0 was used to design upstream and downstream primers to amplify fragments containing rs1801133, rs1801131, and rs1801394 as the targets. The primers and corresponding information are listed in Table 1.

The molecular beacon is a single-stranded stem-loop structure that encompasses a probe region (loop) complementary to the target DNA. The beacon is labeled at the 5′ end with a fluorophore, and at the 3′ end, it carries a quencher. The probes are designed according to the target gene sequence and the site information of the mutation site. All molecular beacon probe sequences and corresponding

TABLE 1 Nucleotide sequences of amplification primers

Target allele	Primers	Sequence of primers (5′–3′)	Tm (°C)	Size
MTHFR C677T	F-1	5′-TGAAGCACTTGAAGGAGAAGGTG-3′	61.0	67bp
	R-1	5′-GCCTCAAAGAAAAGCTGCGTG-3′	60.5	
MTHFR A1298C	F-2	5′-AGGAGGAGCTGCTGAAGATGT-3′	58.3	91bp
	R-2	5′-GGTTTGGTTCTCCCGAGAGGTA-3′	58.5	
MTRR A66G	F-3	5′-CAGCAGGGACAGGCAAAGG-3′	59.8	68bp
	R-3	5′-AGATCTGCAGAAAATCCATGTACCAC-3′	59.3	

targets are listed in Table 2. The Tm values of the probes were predicted using Tm Utility v1.3 software.

2.5 | PCR amplification

The PCR was performed in a total volume of 20 μL, with 2.0 μL of template DNA (50 ng/μL), 2.0 U of Taq DNA polymerase, 2.0 mmol/L MgCl2, 1× PCR buffer, 0.2 mmol/L dNTPs, 0.1 U of UNG enzyme, 0.06 μmol/L forward primer, 0.2 μmol/L reverse primer, and 0.02 μmol/L probe.

The following PCR procedure was applied: 50°C for 2 minutes; denaturation for 3 minutes at 95°C; 45 cycles of amplification with denaturation at 95°C for 20 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 minutes; and the PCR melting stage. The melting program included three steps: denaturation at 95°C for 2 minutes, renaturation at 40°C for 2 minutes, and probe melting curve analysis via a temperature ramp from 40 to 85°C with a heating rate of 0.04°C/s. The analysis was performed on a Roche Cobas z 480 real-time PCR instrument (Roche). Fluorescence data were converted into melting peaks by plotting the negative derivatives of fluorescence with respect to temperature (−dF/dT) as a function of temperature.

2.6 | Establishing the limit of detection

The limit of detection (LOD) is the lowest actual concentration of analyte in a sample that can be detected consistently with acceptable accuracy (eg, in ≥95% of the tested samples).[14] It is also defined as the lowest concentration that yields no more than one negative result in 20 replicates (ie, positive rate, ≥95%).[15] To establish the LOD of this assay, genomic DNA samples with different mutant genotypes and wild type at concentrations of 1, 2, and 4 ng/μL were verified using the assay we developed, each 20 times. All assays were performed in triplicate. The lowest concentration at which at least 19 of the 20 test results of all samples were positive was the LOD in our study.

2.7 | Sanger sequencing

Among the 500 clinical samples, a total of 94 samples were randomly selected, including 13 wild type and 81 mutants, which were analyzed independently by DNA sequencing to confirm the accuracy of the assay.

3 | RESULTS

3.1 | Melting curve analysis

A typical presentation of melting curves is shown in Figure 1. When analyzing the results, the fluorescence of FAM (channels 465-510) represents rs1801133, a single melting curve peak from 55 to 58°C represents wild-type 677CC, a single melting curve peak from 62 to 65°C represents 677TT homozygote, and double peaks with two melting points represent 677CT heterozygote (Figure 1A). The fluorescence of ROX (channels 540-610) represents rs1801394, a single melting curve peak from 54 to 57°C represents wild-type 66AA, a single melting curve peak from 60 to 63°C represents 66GG homozygote, and double peaks with two melting points represent 66AG heterozygote (Figure 1B). The fluorescence of Cy5 (channels 610-670) represents rs1801131, a single melting curve peak from 51 to 54°C represents wild-type 1298AA, a single melting curve peak from 58 to 61°C represents 1298CC homozygote, and double peaks with two melting points represent 1298AC heterozygote (Figure 1C).

3.2 | LOD

Regarding the detection rate, when the genomic DNA concentration was 1 ng/μL, only some genotypes reached 95%. When the concentration was 2 ng/μL, the detection rate of all genotypes was ≥95%. Therefore, the lowest concentration of genomic DNA of clinical samples that could be detected stably by this assay was 2 ng/μL.

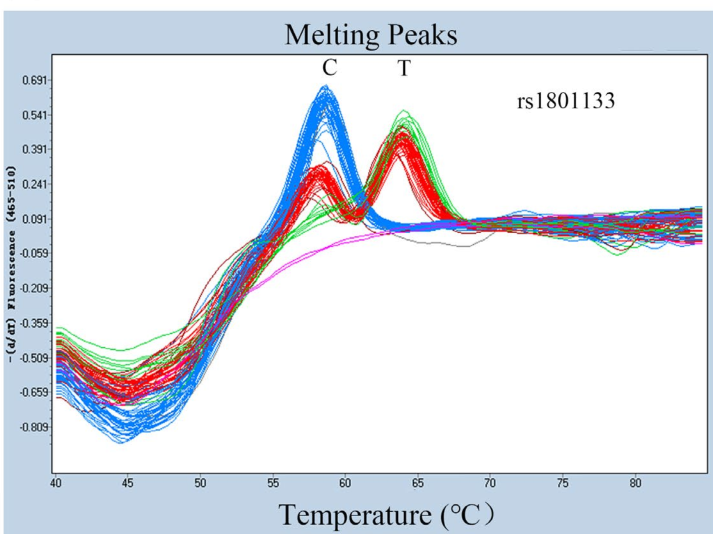
3.3 | Genotyping results of subjects

We applied our method to a group of 500 women of gestational age enrolled in this study. A total of 18 genotypes of these three polymorphisms were identified. Among them, 71 subjects were wild-type in all three polymorphisms; the most common were 677MN, and the other two were wild-type. The polymorphisms of MTHFR and MTRR in 500 subjects of this study are shown in Table 3. The total frequencies of the MTHFR C677T and A1298C and MTRR A66G genotypes

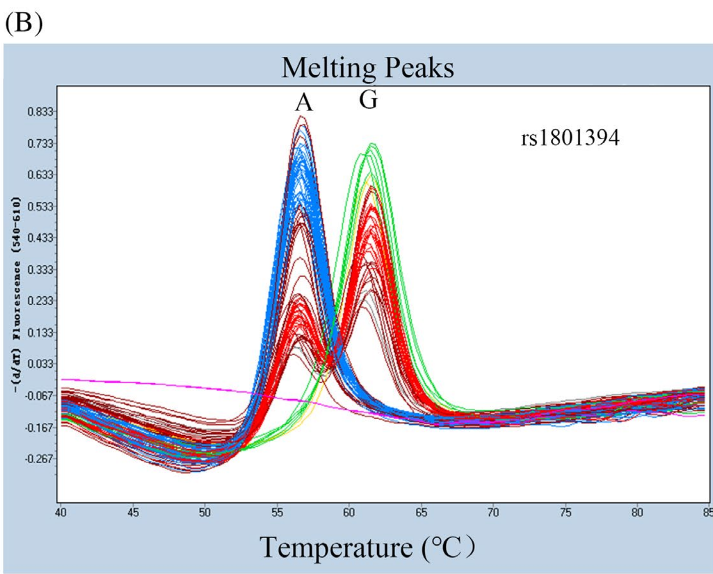
TABLE 2 Nucleotide sequences of the probes

			Tm (°C)	
Probes	Allele	Sequences	Wild type	Mutation type
FAHP1	C677T	FAM-CGGCGCCTGCGGGAGTCGATTTCATCGCGCCG-Dabcy1	55-58	62-65
FAHP2	A1298C	CY5-CGACCAGTGAAGCAAGTGGGTCG-Dabcy1	51-54	58-61
FAHP3	A66G	ROX-GACGAAGAAATGTGTGAGCACGTC-Dabcy1	54-57	60-63

(A)



(B)



(C)

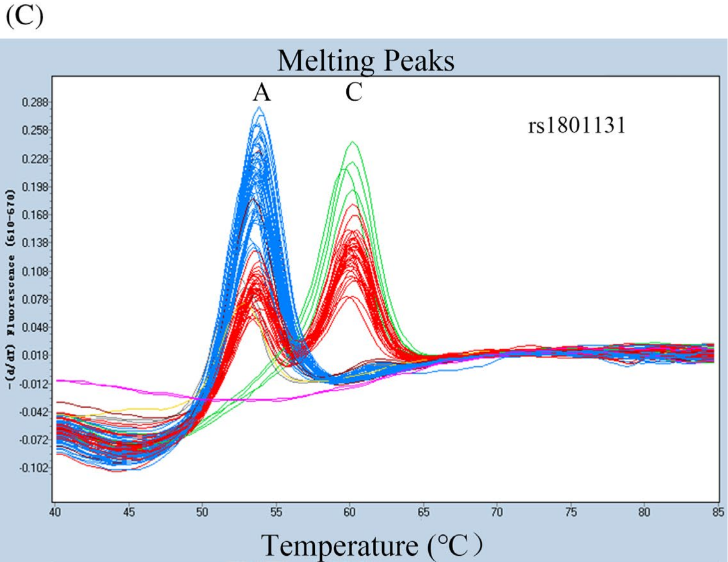


FIGURE 1 A typical presentation of melting curves. (A) 677CT heterozygous type, (B) 66AG heterozygous type, and (C) 1298AC heterozygous type

and alleles are shown in Table 4. The representative results of the samples are shown in Figure 2.

3.4 | Validation of the assay

A total of 94 samples randomly selected from the 500 samples, including 13 with wild type and 81 with mutants, were subsequently examined by DNA sequencing, and 100% concordance was found between the two methodologies. Therefore, both the sensitivity and specificity of the assay were 100% for our study population.

4 | DISCUSSION

At present, the main methods used to detect MTHFR and MTRR gene polymorphisms are PCR restriction fragment length polymorphism (PCR-RFLP) analysis, gene chip analysis, direct sequencing analysis, etc.[16-18] The simplest method is PCR-RFLP, but the operation of this method is complex; the samples are prone to cross contamination of PCR products and to false-negative or false-positive results due to insufficient or excessive enzyme digestion, which indicates low reliability.[19] As the gold standard, Sanger sequencing is highly accurate for detecting polymorphisms but has several limitations, including long sequencing/diagnosis time, high cost, and complex operation and result analysis. Additionally, in Sanger sequencing, a single reaction can only detect one polymorphism.[20] Therefore, it is necessary to establish a method with good specificity and high sensitivity that can detect three polymorphisms in a single reaction tube at the same time.

This study demonstrated that the molecular-beacon-based asymmetric PCR assay we established is an efficient and accurate method to detect the three polymorphisms of MTHFR and MTRR simultaneously. It has several advantages over methods using conventional PCR. There is no need to manipulate PCR products, and all the reactions are performed in a closed system, which reduces the test time, labor, and risk of contamination. The characteristics of a molecular beacon are low background signal, high sensitivity, high specificity, simple operation, and detection without isolation of unreacted probes. In our experience, the whole test process can be completed within 3 hours, and the interpretation of the result was simple and direct.

Our data showed that the frequencies of the 677T allele and the 677TT genotype in the Dongguan region of Guangdong Province were 29.1% and 9.2%, respectively, which agrees with those previously reported among Guangdong Province of 28.5% and 8.3%, respectively.[21] However, they are significantly lower than those of the whole Chinese Han population of 45.2% and 23.2%, respectively ($P < .01$).[21] This is consistent with previous results that the 677T allele and the 677TT genotype frequencies steadily increased from southern to northern China, as Dongguan is located in southern China.[9,21]

For the MTHFR A1298C polymorphism, the frequencies of the 1298C allele and 1298CC genotype were 20.1% and 4.6%, respectively, which were similar to the mean average of the Chinese Han nationality.[21] The distribution of the MTRR A66G polymorphism is less understood. To date, data on the prevalence of the A66G polymorphism in China are limited. Previous studies of Chinese populations showed that the 66G allele and 66GG genotype frequencies ranged from 20%-31% to 2%-8%, respectively.[22-25] In our study, the frequencies of the 66G allele and 66GG genotype were 28.9% and 8.2%, respectively, which are much higher than those of northern China, such as Shandong, Henan, and Shanxi.[21]

Individuals with such genetic mutations in folate-related enzymes cannot meet the body's needs if they supplement folate

TABLE 3 Polymorphisms of MTHFR and MTRR in 500 subjects of this study

Allele			
C677T	A1298C	A66G	No.
MN	N	N	77
N	N	N	71
MN	N	MN	57
N	N	MN	48
N	MN	MN	48
N	MN	M	45
MN	MN	N	28
M	N	N	22
MN	MN	MN	21
M	N	MN	21
N	N	M	12
N	M	MN	12
MN	N	M	11
N	M	N	9
N	MN	N	8
MN	MN	M	5
M	N	M	3
N	M	M	2

TABLE 4 The frequencies of MTHFR C677T, A1298C, and MTRR A66G genotypes and alleles

	Genotype, n (%)						Allele frequency (%)			
	Wild type		Heterozygote		Homozygote					
MTHFR C677T	CC	255 (51.0%)	CT	199 (39.8%)	TT	46 (9.2%)	C	70.9%	T	29.1%
MTHFR A1298C	AA	322 (64.4%)	AC	155 (31.0%)	CC	23 (4.6%)	A	79.9%	C	20.1%
MTRR A66G	AA	252 (50.4%)	AG	207 (41.4%)	GG	41 (8.2%)	A	71.1%	G	28.9%

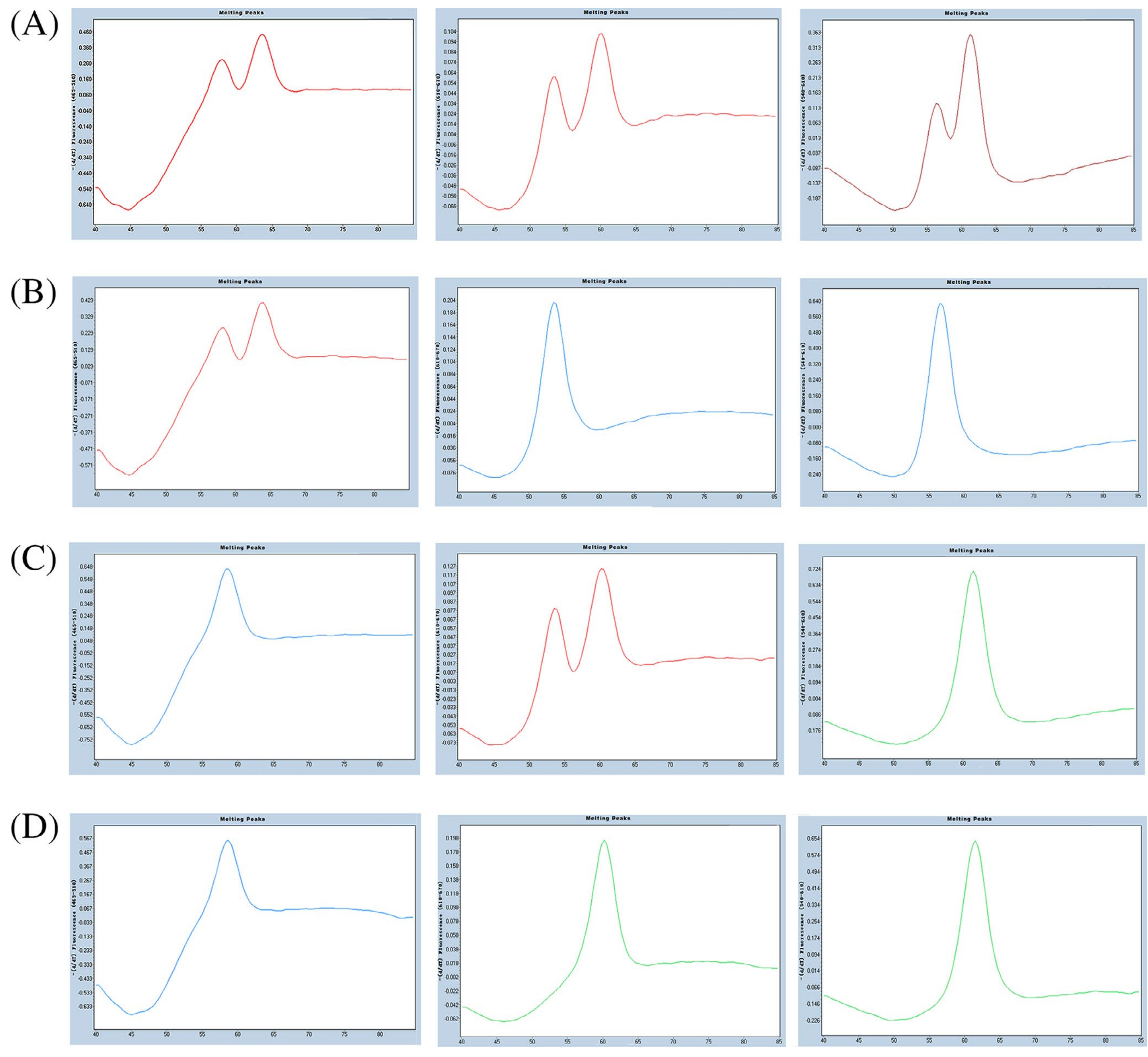


FIGURE 2 Representative results of samples. (A) 677CT, 1298AC, and 66AG; (B) 677CT, 1298AA, and 66AA; (C) 677CC, 1298AC, and 66GG; (D) 677CC, 1298CC, and 66GG

regularly. On the other hand, excessive supplementation with folic acid will also reduce the body's immunity and increase the risk of infection, even in pregnant women, which is related to the risk of neonatal asthma and increased insulin resistance. According to the genotyping results, we can detect the gene polymorphisms of the key enzymes in folate metabolism. The ability of folic acid utilization was graded to achieve individualized folic acid supplementation and to reduce the incidence of birth defects and diseases related to folic acid metabolism.

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