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Genetic polymorphisms of key enzymes in folate metabolism affect the efficacy of folate therapy in patients with hyperhomocysteinaemia

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Abstract

The aim of this study is to analyse the efficacy rate of folate for the treatment of hyperhomocysteinaemia (HHcy) and to explore how folate metabolism-related gene polymorphisms change its efficacy. This study also explored the effects of gene–gene and gene–environment interactions on the efficacy of folate. A prospective cohort study enrolling HHcy patients was performed. The subjects were treated with oral folate (5 mg/d) for 90 d. We analysed the efficacy rate of folate for the treatment of HHcy by measuring homocysteine (Hcy) levels after treatment. Unconditioned logistic regression was conducted to analyse the association between SNP and the efficacy of folic acid therapy for HHcy. The efficacy rate of folate therapy for HHcy was 56·41 %. The *MTHFR* rs1801133 CT genotype, TT genotype and T allele; the *MTHFR* rs1801131 AC genotype, CC genotype and C allele; the *MTRR* rs1801394 GA genotype, GG genotype and G allele; and the *MTRR* rs162036 AG genotype and AG + GG genotypes were associated with the efficacy of folic acid therapy for HHcy ($P < 0{\cdot}05$). No association was seen between other SNP and the efficacy of folic acid. The optimal model of gene–gene interactions was a two-factor interaction model including rs1801133 and rs1801394. The optimal model of gene–environment interaction was a three-factor interaction model including history of hypertension, history of CHD and rs1801133. Folate supplementation can effectively decrease Hcy level. However, almost half of HHcy patients failed to reach the normal range. The efficacy of folate therapy may be genetically regulated.

Key words: Hyperhomocysteinaemia: Folate: Efficacy: Gene polymorphisms

Homocysteine (Hcy), a sulphur-containing amino acid, is produced as an important intermediary of methionine metabolism[1]. The fasting plasma Hcy levels in healthy adults are 5–15 μmol/l, and higher than 15 μmol/l is considered to be hyperhomocysteinaemia (HHcy). In recent years, many studies have supported the association of HHcy with an increased risk of CVD[2]. HHcy has also been linked to increased risks of neural tube defects[3], Alzheimer's disease[4], pregnancy complications[5,6] and inflammatory bowel disease[7].

Factors affecting plasma Hcy level include age, sex, smoking, drinking, nutritional factors, liver and kidney function and folate/Hcy metabolism-related gene polymorphisms, and both genetics and folic acid intake are important[8–12]. In the past two decades, many studies have been conducted to explore the decrease in plasma Hcy levels after folate supplementation[13–15] However, these studies only report the reduction in plasma Hcy levels after long-term folate supplementation and do not report whether plasma Hcy was reduced to a normal level (5–15 μmol/l).

There is no report on the relationship between gene polymorphism and the efficacy of folate therapy in patients with HHcy. In this study, we gave folate supplements to patients with HHcy at a sufficient dose and reported the efficacy of folate therapy to reduce plasma Hcy levels to normal. The subjects were divided into the failure group and the success group according to the plasma Hcy level after the intervention. The relationships between environmental factors and folate metabolism-related gene polymorphisms and the efficacy of folate on HHcy were explored. This study provides a scientific basis for more effective prevention and treatment of HHcy.

Methods

Study design and participants

We performed a prospective cohort study of HHcy patients (Hcy ≥ 15 μmol/l) who had plasma Hcy levels measured in the Department of Neurology in the Fifth Affiliated Hospital of Zhengzhou University from July to December 2014. The

Abbreviations: Hcy, homocysteine; HHcy, hyperhomocysteinaemia.

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subjects included in our study met the following criteria: (1) tested for the plasma Hcy level, (2) diagnosed with HHcy (total plasma Hcy ≥ 15 μmol/l), (3) >18 years of age and (4) voluntarily participated in this study and received 90 d of folic acid supplementation. Subjects with a history of serious infection, hepatic or kidney diseases, haematologic disorders or cancer and those who used vitamin B or folic acid supplements or use medications that interfere with folate metabolism (methotrexate, phenytoin, etc.) in the prior 2 weeks were excluded. Thus, a total of 858 subjects were ultimately included in this study. Fasting plasma Hcy levels were measured at day 1. Then, the enrolled patients were treated with oral folic acid (5 mg/d) for 90 d. The compliance with oral folic acid was assessed by telephone interview at 45 and 90 d of follow-up. Plasma Hcy levels were obtained at the second follow-up.

The study was approved by the Ethics Review Committee of the Life Science of Zhengzhou University. All subjects or relatives signed informed consent.

Efficacy criteria and grouping standard

The therapy was effective if patients' Hcy levels decreased to 15 μmol/l or less, which put them in the success group. The therapy was unsuccessful if patients' Hcy levels were ≥15 μmol/l, which put them in the failure group.

Patients were also grouped according to their baseline plasma Hcy level. Baseline Hcy levels with elevations of 16–30, 31–100 and > 100 μmol/l were classified as mild, moderate and severe HHcy, respectively[16].

Blood collection and laboratory techniques

At baseline, overnight-fasting blood samples were drawn for the clinical chemistry tests and for the measurements of plasma Hcy, which were also measured at the end of study period. The remaining samples were immediately placed on ice, transported to the molecular biology laboratory and centrifuged at 12 000 rpm for 5 min. Plasma and cells were separated and stored at −80°C until analysis. Genomic DNA was extracted using a whole-blood genomic DNA extraction kit (BioTeke®). DNA extraction was performed according to the manufacturer's instructions.

Sample size

According to the relevant references, and with the effects of folic acid supplementation and folate-related enzyme gene polymorphisms on plasma Hcy level taken into consideration, a nonparametric matching design was adopted. The SNP with the lowest variation rate of genetic loci in the population was selected to estimate the sample size. Minor allele frequency (MAF) $P_0 = 13\%$, expected risk ratio (RR) = 2·0, $\alpha = 0{\cdot}05$ and $\beta = 0{\cdot}10$. The formula is:

$$n = 2pq(Z\alpha + Z\beta)^2/(P_1 - P_0)^2$$

$$P_1 = P_0\mathrm{RR}/[1 + P_0(\mathrm{RR} - 1)]$$

$$\overline{p} = 0{\cdot}5(P_1 + P_0); \overline{q} = 1 - \overline{p}.$$

The sample size was approximately 300, with 150 in each group.

DNA quantification and purity determination

The purity of the extracted DNA solution was measured by an ultramicroscopic UV-V is spectrophotometer. The ratios of A260:A280 and A260:A230 were determined at pH 7–8·5. The purity of DNA was determined by the ratio of A260:A280; a ratio between 1·8 and 2·0 indicated a higher DNA purity, and a ratio <1·8 indicated contamination by protein or phenolic substances.

SNP selection and genotyping

HaploView4.2 software was used to download the information on *MTRR*, *MTR*, *MTHFR* and *MTHFD* SNP in the Chinese Han population from HapMap. Selection criteria were as follows: MAF > 0·05; linkage disequilibrium value $r^2 > 0{\cdot}8$; and functional SNP or SNP that induces a change in protein activity.

We selected three SNP in *MTRR*: rs1801394, rs162036 and rs1532268; three SNP in *MTR*: rs1805087, rs1266164 and rs12354209; three SNP in *MTHFR*: rs1801133, rs1801131 and rs2274976; and two SNP in *MTHFD*: rs2236225 and rs1950902. Genotypes and alleles were detected using Sequenom's MassArray system.

Statistical analysis

Statistical analyses were conducted using the SPSS software package (version 21.0 for windows). Patients who were lost to follow-up or had poor compliance were excluded. The numerical data were expressed as median values with interquartile ranges (25th–75th percentiles). Changes in Hcy level from pre-treatment to post-treatment were compared by Student's *t* test. The relative decrease in Hcy levels is expressed as (Hcy after treatment − Hcy at baseline)/(Hcy at baseline). The difference in the efficacy rate was assessed by the χ^2 test. The significance of any differences between the two groups was examined with Student's *t* test or the χ^2 test.

The relationship between SNP and the efficacy of folic acid therapy for HHcy were examined using unconditioned logistic regression models with and without adjustment for smoking, drinking, history of diabetes, hypertension, CHD and biochemical indicators. The IHG webpage's online detection method was used to analyse Hardy–Weinberg equilibrium. SHEsis online software was used to estimate haplotypes. Multifactor dimensionality reduction (MDR) software was used to evaluate gene–gene and gene–environment interactions. We considered a two-tailed *P* value < 0·05 as significant. The statistical power was calculated by the standard formula.

Results

Efficacy of folic acid treatment for hyperhomocysteinaemia

A total of 1033 patients with HHcy were enrolled at the baseline, and 175 patients who were lost to follow-up or had poor compliance were excluded. A total of 858 were ultimately included in this study. The average age of the patients was 64·74 (SD 13·34) years. After treatment with folic acid for 90 d, the plasma Hcy levels of 484 patients decreased to 15·0 μmol/l or less, corresponding to an efficacy rate of 56·41 %.

Before treatment, the median value of the plasma Hcy levels was 22·10 (SD 8·55) μmol/l. After treatment, the value was 15·90 (SD 5·74) μmol/l. The relative decrease in plasma Hcy levels was 28·05 % overall. Plasma Hcy levels after treatment were significantly lower than those before treatment ($t = 28·41$, $P < 0·05$).

Table 1 shows the distribution of demographic features and plasma Hcy baseline levels. Table 2 shows the general characteristics of the success group and failure group. Furthermore, baseline Hcy level had an effect on the efficacy of folate therapy in patients with HHcy (Table 3).

Association of MTHFR *gene polymorphism with the efficacy of folate*

The frequency distributions of the genotypes and alleles of the *MTHFR* rs1801133, rs1801131 and rs2274976 SNP in the failure group and the success group are shown in Table 4.

Table 1. Distribution of demographic features and plasma homocysteine (Hcy) baseline levels
(Numbers and percentages; mean values and standard deviations)

Characteristics	*n*	%
Number of individuals	858	
Age (years)		
Mean	64·74	
SD	13·34	
Female	322	37·5
Diseases		
Stroke	444	51·7
Transient ischaemic attack	22	2·6
Vertebral-basilar artery insufficiency	151	17·6
Posterior circulation ischaemia	159	18·5
Other diseases	82	9·6
Baseline plasma Hcy (μmol/l)		
Mean	22·10	
SD	8·55	

The frequency distributions of rs1801133 and rs1801131 genotypes and alleles were significantly different between the failure group and the success group after adjustment for age, sex, smoking, drinking and biochemical parameters by the binary logistic regression analysis ($P < 0·05$). There was no significant difference in rs2274976 genotype or allele frequency between the failure group and success group, which indicates that rs1801131 and rs1801133 were associated with the effects of folic acid on HHcy, and rs2274976 was not.

The TT genotype and CT + TT genotypes of the rs1801133 SNP made up 47·29 and 89·14 % of the failure group, respectively, which were significantly higher than those in the success group (33·85 and 77·85 %) ($P < 0·05$). Compared with the CC genotype, the risk of treatment failure was 2·68 times ($P < 0·000$, OR = 2·68; 95 % CI = 1·59, 4·54) and 2·12 times ($P = 0·003$, OR = 2·12; 95 % CI 1·3, 3·44) as high in individuals carrying the TT genotype and CT + TT genotypes, respectively. The frequency of the T allele in the failure group was 68·21 %, which was significantly higher than the 55·85 % in the success group ($P < 0·05$). Taking the C allele as a control, the risk of treatment failure was 1·69 times ($P < 0·000$, OR = 1·69; 95 % CI 1·35, 2·13) as high in individuals carrying the T allele.

The genotype and allele of rs1801131 were significantly different between the failure group and the success group ($P < 0·05$). Compared with the AA genotype, the AC genotype and the CC genotype could decrease the risk of failure of folic acid therapy for HHcy (OR = 0·52; 95 % CI 0·33, 0·81) and 0·26

Table 3. Effect of the baseline homocysteine (Hcy) level on the efficacy of folate treatment for hyperhomocysteinaemia (HHcy)

Baseline Hcy level grouping	Success group	Failure group	Efficacy rate (%)	χ^2	*P*
Mild HHcy	449	283	61·34	49·24	< 0·001
Moderate to severe HHcy	35	91	27·78		

Table 2. General characteristics of success group and failure group
(Mean values and standard deviations)

	Success group (*n* 325)		Failure group (*n* 313)			
Variables	Mean	SD	Mean	SD	χ^2/t	*P*
Age (years)	64·6	15·8	66·2	13·4	−1·43*	0·152
Sex (male/female)	183/142		199/114		3·51	0·061
Smoking (yes/no)	98/227		121/192		5·12	0·024
Alcohol consumption (yes/no)	42/283		51/262		1·46	0·228
Baseline plasma Hcy levels (μmol/l)	20·2	6·4	24·3	9·7	−6·30*	<0·001
FPG (mmol/l)	5·41	1·94	5·63	2·21	−1·36*	0·174
TC (mmol/l)	4·23	1·00	4·47	0·99	−3·08*	0·003
TAG (mmol/l)	1·53	1·04	1·63	1·21	−1·13*	0·261
HDL-cholesterol (mmol/l)	1·15	0·32	1·07	0·27	3·47*	0·001
LDL-cholesterol (mmol/l)	2·45	0·73	2·65	0·75	−3·51*	<0·001
Past history						
Diabetics (yes/no)	51/274		110/203		31·98	<0·001
Hypertension (yes/no)	145/180		208/105		30·77	<0·001
Stroke (yes/no)	127/198		139/174		1·87	0·172
Hyperlipidaemia (yes/no)	5/320		12/301		3·24	0·072
CHD (yes/no)	42/283		122/191		56·68	<0·001

Hcy, homocysteine; FPG, fasting plasma glucose; TC, total cholesterol.
* *t* Value, not χ^2.

Table 4. Association of *MTHFR* gene polymorphism with the efficacy of folate treatment for hyperhomocysteinaemia (Numbers and percentages; odds ratios and 95 % confidence intervals)

	Success group		Failure group										
Genotypes	*n*	%	*n*	%	χ^2	*P*	Crude OR	95 % CI	χ^2	*P*	Adjusted OR	95 % CI	Failure rate (%)
rs1801133													
CC	72	22·15	34	10·86			Ref.				Ref.		32·07
CT	143	44·00	131	41·85	7·58	0·006	1·94	1·21, 3·11	3·90	0·048	1·69	1·00, 2·85	47·81
TT	110	33·85	148	47·29	18·54	0·000	2·85	1·77, 4·59	13·51	0·000	2·68	1·59, 4·54	57·36
CT + TT	253	77·85	279	89·14	14·67	0·000	2·34	1·50, 3·63	9·09	0·003	2·12	1·30, 3·44	52·44
C	287	44·15	199	31·79			Ref.						40·95
T	363	55·85	427	68·21	20·68	0·000	1·69	1·35, 2·13					54·05
rs1801131													
AA	232	71·39	255	81·47			Ref.				Ref.		52·36
AC	82	25·23	54	17·25	6·74	0·009	0·60	0·41, 0·88	8·43	0·004	0·52	0·33, 0·81	39·71
CC	11	3·38	4	1·28	3·50	0·061	0·33	0·10, 1·05	4·44	0·035	0·26	0·07, 0·91	26·67
AC + CC	93	28·62	58	18·53	8·865	0·003	0·57	0·39, 0·82	11·19	0·001	0·48	0·32, 0·74	38·41
A	546	84·00	564	90·10			Ref.						50·81
C	104	16·00	62	9·90	10·47	0·001	0·58	0·41, 0·81					37·35
rs2274976													
GG	301	92·62	290	92·65			Ref.				Ref.		49·07
GA	24	7·38	23	7·35	0·00	0·986	0·99	0·55, 1·80	0·44	0·508	0·80	0·41, 1·56	48·94
G	626	96·31	603	96·33			Ref.						49·06
A	24	3·69	23	3·67	0·00	0·986	0·99	0·56, 1·78					48·94

Ref., referent values.

Table 5. Association of *MTHFD* gene polymorphism with the efficacy of folate treatment for hyperhomocysteinaemia (Numbers and percentages; odds ratios and 95 % confidence intervals)

	Success group		Failure group										
Genotypes	*n*	%	*n*	%	χ^2	*P*	Crude OR	95 % CI	χ^2	*P*	Adjusted OR	95 % CI	Failure rate (%)
rs2236225													
CC	185	56·92	191	61·02			Ref.				Ref.		50·80
CT	121	37·23	105	33·55	1·06	0·303	0·84	0·60, 1·17	0·15	0·703	0·93	0·65, 1·34	46·46
TT	19	5·85	17	5·43	0·17	0·682	0·87	0·44, 1·72	0·06	0·815	0·91	0·43, 1·96	47·22
CT + TT	140	43·08	122	38·98	1·11	0·293	0·84	0·62, 1·16	0·17	0·680	0·93	0·65, 1·32	46·56
C	491	79·54	487	77·80			Ref.						49·80
T	169	24·46	139	22·20	0·91	0·341	0·88	0·68, 1·14					45·13
rs1950902													
CC	166	51·08	186	59·42			Ref.				Ref.		52·84
CT	137	42·15	109	34·82	4·21	0·040	0·71	0·51, 0·99	3·61	0·058	0·70	0·49, 1·01	44·31
TT	22	6·77	18	5·75	0·88	0·348	0·73	0·38, 1·41	2·56	0·133	0·57	0·27, 1·19	45·00
CT + TT	159	48·92	127	40·58	4·50	0·034	0·71	0·52, 0·98	4·60	0·032	0·68	0·48, 0·97	44·41
C	469	72·15	481	76·84			Ref.						50·63
T	181	27·85	145	23·16	3·67	0·060	0·78	0·61, 1·01					44·48

Ref., referent values.

(95 % CI 0·07, 0·91), respectively). Individuals with the C allele favoured folic acid therapy for HHcy (OR = 0·58; 95 % CI 0·41, 0·81), with the A allele as control.

Association of MTHFD *gene polymorphism with the efficacy of folate*

The frequency distributions of genotypes and alleles of the *MTHFD* rs2236225 and rs1950902 SNP in the failure group and the success group are shown in Table 5. By unconditioned logistic regression analysis, we did not find that the frequency distribution of genotypes and alleles of rs2236225 were significantly different between the failure group and the success group and only found that the frequency distribution of the CT + TT genotypes of rs1950902 was statistically significantly different between the failure group and the success group. These findings suggest that rs2236225 has nothing to do with the efficacy of folic acid treatment on HHcy and that rs1950902 is weakly correlated with the efficacy of folic acid treatment on HHcy.

Association of MTR *gene polymorphism with the efficacy of folate*

The frequency distribution of the genotypes and alleles of the *MTR* SNP rs1805087, rs1266164 and rs12354209 in the failure group and the success group are shown in Table 6. By unconditioned logistic regression analysis, after adjustment for age, sex, smoking, drinking, personal disease history, biochemical indexes and other factors, the genotypes and alleles of rs1805087, rs1266164 and rs12354209 in the failure group and the success group were not statistically significantly different, which suggested that rs1805087, rs1266164 and rs12354209 had no effect on HHcy treatment with folic acid.

Table 6. Association of *MTR* gene polymorphism with the efficacy of folate treatment for hyperhomocysteinaemia (Numbers and percentages; odds ratios and 95 % confidence intervals)

	Success group		Failure group										
Genotypes	*n*	%	*n*	%	χ^2	*P*	Crude OR	95 % CI	χ^2	*P*	Adjusted OR	95 % CI	Failure rate (%)
rs1805087													
AA	269	82·77	259	82·75			Ref.				Ref.		49·05
AG	54	16·61	49	15·65	0·08	0·783	0·94	0·62, 1·44	0·20	0·653	0·90	0·56, 1·44	47·57
GG	2	0·62	5	1·60	1·29	0·257	2·60	0·50, 13·50	1·88	0·171	3·44	0·59, 20·22	71·43
AG + GG	56	17·23	54	17·25	0·00	0·994	1·00	0·66, 1·51	0·01	0·907	0·97	0·62, 1·54	49·09
A	592	91·08	567	90·58			Ref.						48·92
G	58	8·92	59	9·42	0·10	0·756	1·06	0·73, 1·55					50·43
rs1266164													
GG	203	62·46	185	59·11			Ref.				Ref.		47·68
GA	109	33·54	110	35·14	0·36	0·546	1·12	0·80, 1·54	0·50	0·480	1·14	0·79, 1·65	50·23
AA	13	4·00	18	5·75	1·23	0·268	1·52	0·72, 3·19	2·48	0·115	1·93	0·85, 4·38	58·06
GA + AA	122	37·54	128	40·89	0·75	0·385	1·51	0·84, 1·58	1·20	0·274	1·22	0·86, 1·74	51·20
G	515	79·23	480	76·68			Ref.						48·24
A	135	20·77	146	23·32	1·21	0·271	1·16	0·89, 1·51					51·96
rs12354209													
AA	109	33·54	119	38·02			Ref.				Ref.		52·19
GA	172	52·92	152	48·56	1·49	0·222	0·81	0·58, 1·14	0·70	0·404	0·85	0·58, 1·24	46·91
GG	44	13·54	42	13·42	0·28	0·596	0·87	0·53, 1·44	0·03	0·863	0·95	0·55, 1·66	48·84
GA + GG	216	66·46	194	61·98	1·39	0·238	0·82	0·60, 1·14	0·55	0·457	0·87	0·61, 1·25	47·32
A	390	60·00	390	62·30			Ref.						50·00
G	260	40·00	236	37·70	0·71	0·399	0·91	0·73, 1·14					47·58

Ref., referent values.

Table 7. Association of *MTRR* gene polymorphism with the efficacy of folate treatment for hyperhomocysteinaemia (Numbers and percentages; odds ratios and 95 % confidence intervals)

	Success group		Failure group										
Genotypes	*n*	%	*n*	%	χ^2	*P*	Crude OR	95 % CI	χ^2	*P*	Adjusted OR	95 % CI	Failure rate (%)
rs1801394													
AA	186	57·23	111	35·46			Ref.				Ref.		37·37
AG	120	36·92	174	55·59	27·69	0·000	2·43	1·75, 3·38	26·35	0·000	2·66	1·83, 3·85	59·18
GG	19	5·85	28	8·95	8·00	0·005	2·47	1·32, 4·63	8·93	0·003	2·88	1·44, 5·76	59·57
AG + GG	139	42·77	202	64·54	30·36	0·000	2·44	1·77, 3·35	28·85	0·000	2·68	1·87, 3·85	59·24
A	492	75·69	396	63·26			Ref.						44·59
G	158	24·31	230	36·74	23·30	0·000	1·81	1·42, 2·30					59·28
rs162036													
AA	217	66·77	237	75·72			Ref.				Ref.		52·20
AG	98	30·15	61	19·49	8·92	0·003	0·57	0·39, 0·82	8·06	0·005	0·56	0·37, 0·83	38·36
GG	10	3·08	15	4·79	0·57	0·449	1·37	0·60, 3·12	0·65	0·420	1·47	0·58, 3·76	60·00
AG + GG	108	33·23	76	24·28	6·22	0·013	0·64	0·46, 0·91	5·62	0·018	0·63	0·43, 0·92	41·30
A	532	81·85	535	85·46			Ref.				Ref.		50·14
G	118	18·15	91	14·54	3·05	0·081	0·77	0·57, 1·03					43·54
rs1532268													
GG	235	72·31	209	66·77			Ref.				Ref.		47·07
GA	87	26·77	96	30·67	1·50	0·220	1·24	0·88, 1·75	1·52	0·218	1·27	0·87, 1·89	52·46
AA	3	0·92	8	2·56	2·58	0·108	3·00	0·79, 11·45	3·77	0·052	4·37	0·99, 16·51	72·73
GA + AA	90	27·69	104	33·23	2·31	0·129	1·30	0·93, 1·82	2·56	0·110	1·36	0·93, 1·99	53·60
A	557	85·69	514	82·11			Ref.						47·99
G	93	14·31	112	17·89	3·04	0·081	1·31	0·97, 1·76					54·63

Ref., referent values.

Association of MTRR *gene polymorphism with the efficacy of folate*

The frequency distribution of genotypes and alleles of the *MTRR* SNP rs1801394, rs162036 and rs1532268 in the failure group and the success group are shown in Table 7. By unconditioned logistic regression analysis, after adjustment for age, sex, smoking, drinking, personal disease history, biochemical indexes and other factors, there were significant differences in the genotypes and alleles of rs1801394 and the rate of the AG + GG genotypes of rs162036 between the failure group and the success group, but there was no significant difference in the genotypes and alleles of rs1532268, which suggested that rs1801394 and rs162036 are associated with the efficacy of folic acid treatment for HHcy, but rs1532268 is not.

The frequency distribution of the AG, GG and AG + GG genotypes of rs1801394 in the failure group were 55·59, 8·95 and

64·54 %, respectively, which were significantly higher than those in the success group (36·92, 5·85 and 42·77 %) ($P<0·05$). Compared with the AA genotype, the risk of folic acid treatment of HHcy failure in individuals carrying the AG genotype, GG genotype and AG + GG genotypes was 2·66 times ($P<0·000$, OR = 2·66; 95 % CI 1·83, 3·85), 2·88 times ($P=0·003$, OR = 2·88; 95 % CI 1·44, 5·76) and 2·68 times ($P<0·000$, OR = 2·68; 95 % CI 1·87, 3·85) as high as that for individuals carrying the CC genotype. The frequency of the G allele in the failure group was 36·74 %, which was significantly higher than that of the success group (24·31 %, $P<0·05$). The risk of treatment failure in individuals carrying the G allele was 1·81 times as great as in individuals carrying the A allele ($P=0·000$, OR = 1·81; 95 % CI 1·42, 2·30).

The frequencies of the AG genotype and AG + GG genotypes of rs162036 in the failure group were 19·49 and 24·28 %, respectively, which were significantly lower than those in the success group (30·15 and 33·23 %, $P<0·05$). Compared with the AA genotype, the individuals with the AG genotype and the AG + GG genotypes favoured folic acid therapy for HHcy (OR = 0·56; 95 % CI 0·37, 0·83 and OR = 0·63; 95 % CI 0·43, 0·92, respectively). Individuals with the G allele were favourably treated with folic acid, but there was no significant difference ($P>0·05$) from the A allele as control.

Association of MTHFR *and* MTRR *gene haplotypes with the efficacy of folate*

The linkage disequilibrium parameters (r^2) of *MTHFR* rs1801133 and rs1801131 and *MTRR* rs1801394 and rs162036 were 0·96 and 0·75, respectively, which suggested that there was a linkage disequilibrium between the two.

The haplotypes of *MTHFR* rs1801133-rs1801131 and *MTRR* rs1801394-rs162036 were analysed. The frequency distribution of each haplotype in the success and failure groups are shown in Tables 8 and 9. The haplotype analysis showed that rs1801133 and rs1801131 produced four haplotypes. The frequencies of the CA and CC haplotypes in the failure group were lower than those in the success group ($P<0·05$). These results show an association with risk of folic acid treatment failure. The frequency of the TA haplotype was significantly higher in the failure group than in the success group ($P<0·05$), which suggested that the TA haplotype could increase the risk of failure of folic acid therapy for HHcy.

rs1801394 and rs162036 also produced four haplotypes. The frequency of the AG haplotype in the failure group was lower than that in the success group ($P<0·05$), which suggested that the AG haplotype could reduce the risk of folic acid treatment failure for HHcy. The frequency of the GA haplotype in the failure group was significantly higher than that in the success group ($P<0·05$), which suggested that the GA haplotype could increase the risk of failure of folic acid treatment.

Gene–gene interaction

In this study, we analysed the interaction between genes and genes by using multi-factor dimensionality reduction (MDR) software. The polymorphisms of 11 SNP (rs1801133 (X1), rs1801131 (X2), rs2274976 (X3), rs2236225 (X4), rs1950902 (X5), rs1801394 (X6), rs162036 (X7), rs1532268 (X8), rs1805087 (X9), rs1266164 (X10) and rs12354209 (X11)) were introduced

Table 10. Gene–gene interaction
(Odds ratios and 95 % confidence intervals)

Models	Training set balance accuracy	Test set balance accuracy	Cross-validation consistency	OR	95 % CI
X6	0·61	0·58	7/10	2·44	1·77, 3·35
X1, X6	0·62	0·62	10/10	2·65	1·92, 3·65
X1, X6, X8*	0·64	0·56	5/10	3·03	2·18, 4·21

* X1: rs1801133; X6: rs1801394; X8: rs1532268.

Table 8. Association of haplotypes of *MTHFR* rs1801133-rs1801131 with the efficacy of folate treatment for hyperhomocysteinaemia
(Numbers and percentages; odds ratios and 95 % confidence intervals)

	Failure group (2*n* 626)		Success group (2*n* 650)					
Haplotypes	*n*	%	*n*	%	χ^2	*P*	OR	95 % CI
CA	137	21·9	187	28·8	8·31	0·004	0·69	0·53, 0·89
CC	62	9·9	100	15·4	8·93	0·003	0·60	0·43, 0·84
TA	427	68·2	359	55·2	21·49	0·000	1·72	1·36, 2·16
TC			4	0·6				

Table 9. Association of haplotypes of *MTRR* rs1801394-rs162036 with the efficacy of folate treatment for hyperhomocysteinaemia
(Odds ratios and 95 % confidence intervals)

	Failure group (2*n* 626)		Success group (2*n* 650)					
Haplotypes	*n*	%	*n*	%	χ^2	*P*	OR	95 % CI
AA	388	54·0	384	59·1	3·15	0·076	0·82	0·66, 1·02
AG	73	11·7	108	16·6	6·43	0·011	0·66	0·48, 0·91
GA	207	33·0	148	22·8	16·84	0·000	1·68	1·31, 2·15
GG	8	1·3	10	1·5				

into the MDR software in the data format (txt format). The optimal model was obtained by software fitting with the interaction of first- to third-order different gene loci (Table 10).

The optimal factor for the univariate model was rs1801394, which showed that the risk of treatment failure in the high-risk populations with rs1801394 was 2·44 times (OR = 2·44; 95 % CI 1·77, 3·35) as high as in the low-risk populations. The optimal model of the two-factor interaction model was rs1801133 and rs1801394. The results showed that the risk of treatment failure in the high-risk populations with rs1801133 and rs1801394 was 2·65 times as high as in the low-risk group (OR = 2·65; 95 % CI 1·92, 3·65). The optimal model of the three-factor interaction model was rs1801133, rs1801394 and rs1532268. The results showed that the risk of treatment failure in the high-risk populations with rs1801133, rs1801394 and rs1532268 was 3·03 times as high as that of the low-risk group (OR = 3·03; 95 % CI 2·18. 4·21). According to the test set of the first- to third-order optimal model, the higher the value of the equilibrium accuracy and the higher the consistency of cross-validation, the better the model was. In addition, the two-factor interaction model was the best combination model. The effects of the combination of rs1801133 and rs1801394 on the efficacy of folic acid in the treatment of HHcy were more stable.

Gene–environment interaction

Next, we analysed the interaction between genes and environment with MDR. The results showed that smoking, diabetes, hypertension, CHD, total cholesterol (TC), HDL, LDL and other factors were significantly different between the failed group and the successful group (Table 2). With reference to the guideline of prevention and treatment of dyslipidaemia in Chinese adults, the TC, HDL-cholesterol and LDL-cholesterol converted into two categories of variables (TC ≥ 5·18 mmol/l, HDL-cholesterol < 1·04 mmol/l and LDL-cholesterol ≥ 3·37 mmol/l assigned to 1). Then, smoking (X12), drinking (X13), diabetes mellitus (X14), history of hypertension (X15), history of CHD (X16), TC (X17), HDL (X18), LDL (X19) and the polymorphisms of eleven SNP were introduced into the MDR software in the data format (txt format). The optimal model was obtained by MDR software fitting with the interaction of first- to third-order different risk factors (Table 11).

The optimal factor for the univariate model was CHD. The risk of failure of folic acid treatment with HHcy in patients with a history of CHD was 4·36 times (OR = 4·36; 95 % CI 2·59, 4·68) that of patients without CHD. The optimal model of the two-factor interaction model was rs1801394 and history of CHD. The results showed that the risk of treatment failure in the high-risk population with rs1801394 and history of CHD was 4·75 times higher than that of the low-risk group (OR = 4·75; 95 % CI 2·94, 7·08). The optimal model of the three-factor interaction model was rs1801133, history of CHD and history of hypertension. The results showed that the risk of treatment failure in the high-risk population with rs1801133, history of CHD and history of hypertension was 6·88 times higher than that of the low-risk group (OR = 6·88; 95 % CI 3·98, 16·70). According to the optimal model of first to third order, the higher the value of the test set balance accuracy and the higher the consistency of cross-validation, the better the model was. The three-factor interaction model was the best combination model. That is to say, the effects of the combination of rs1801133, history of CHD and history of hypertension on the efficacy of folic acid in the treatment of HHcy were larger and more stable.

Table 11. Gene–environment interaction (Odds ratios and 95 % confidence intervals)

Models	Training set balance accuracy	Test set balance accuracy	Cross-validation consistency	OR	95 % CI
X16	0·63	0·60	6/10	4·36	2·59, 6·48
X6*, X16	0·65	0·62	7/10	4·75	2·94, 7·08
X1, X15, X16*	0·67	0·67	10/10	6·88	3·98, 16·70

* X1: rs1801133; X6: rs1801394; X15: history of hypertension; X16: history of CHD.

Discussion

HHcy is an independent risk factor for cardiovascular diseases, and oral folate can reduce plasma Hcy concentration. However, data on the Hcy-lowering effects of folic acid therapy are limited. In this study, we adopted a prospective method to observe the efficacy of folate therapy and found 484 of 858 HHcy patients (56·41 %) reached normal serum Hcy levels with folate treatment (5 mg/d) for 3 months. The efficiency of folate treatment of HHcy was low. Therefore, we combined the genetic factors and environmental factors to explore the causes of treatment failure.

We found that the CT genotype, TT genotype and T allele of *MTHFR* rs1801133 increased the risk of folate therapy failure for HHcy. Some studies have shown that rs1801131 was independent of plasma Hcy level and had no significant effect on the effect of folic acid on Hcy level[17–20]. This might be related to the distribution of *MTHFR* in different races and different populations. We also found that the AC genotype, the CC genotype and the C allele of *MTHFR* rs1801131 decreased the risk of folic acid therapy failure for HHcy. In addition, the genotype frequency distribution of *MTHFR* rs2274976 was not significantly different between the success group and the failure group and might not be related to the effect of folic acid on HHcy. At present, the majority of studies have shown the associations between *MTHFR* rs2274976 and diseases such as cleft lip, cleft palate and neural tube defects[21–23]. Few studies have reported the effect of *MTHFR* rs2274976 on the efficacy of folic acid supplementation on Hcy level.

The results showed that *MTHFD* rs2236225 and *MTHFD* rs1950902 were not associated with the efficacy of the folic acid therapy on HHcy. In fact, no study has found that rs2236225 or rs1950902 was related to Hcy level. The frequencies of the GG genotype, the AG genotype and the AA genotype of rs2236225 in this healthy population in northern China were 57·98, 35·57 and 6·45 % respectively, and the frequency of the A allele was 24·23 %, which was consistent with the gene distribution of the failure group and successful group. However, another study found that rs2236225 had nothing to do with the pathogenesis of CHD[24]. The interaction between rs2236225 and Hcy may be involved in the occurrence of gastric cancer in the Chinese Han population[25].

There was no relationship between the genotypes and alleles of *MTR* rs1805087 and the efficacy of folic acid on HHcy. Studies have shown that there are significantly different genotype distributions of *MTR* rs1805087 in different regions and different ethnic groups[26]. This might be the reason why the results were inconsistent. This study found that *MTR* rs1266164 and rs12354209 were not associated with the effect of folic acid on HHcy.

MTRR has a key role in the remethylation of Hcy to methionine. We found that the AG genotype, GG genotype and G allele of *MTRR* rs1801394 increased the risk of treatment failure. rs1801394 has been associated with abnormal plasma Hcy levels in the body[27]. It had been found that individuals with the G allele of rs1801394 have a higher risk of developing HHcy than individuals with the A allele by 2·1 times[28]. The AG genotype and AG + GG genotype of rs162036 were associated with the efficacy (OR0·56; 95 % CI 0·37, 0·83 and OR = 0·63; 95 % CI 0·43, 0·92, respectively). These results indicate that mutations of rs162036 could reduce the risk of treatment failure. A previous study found that there was no significant difference in Hcy level between the AA genotype, AG genotype and GG genotype of rs162036[29], a result that might be related to the different types of subjects included in this paper. In this study, rs1532268 was not associated with the efficacy of folate in the treatment of HHcy.

To further explore the association between *MTHFR* or *MTRR* gene polymorphism and the efficacy of folate in the treatment of HHcy, the haplotypes of *MTHFR* rs1801133-rs1801131 and *MTRR* rs1801394-rs162036 were analysed. Our results suggest that the TA haplotype of *MTHFR* rs1801133-rs1801131 and the GA haplotype of *MTRR* rs1801394-rs162036 could increase the risk of failure of folic acid therapy for HHcy. The CA haplotype and CC haplotype of *MTHFR* rs1801133-rs1801131 and the AG haplotype of *MTRR* rs1801394-rs162036 could decrease the risk of failure of folic acid therapy for HHcy. The CA haplotype of rs1801133-rs1801131 was favourable for the treatment of HHcy by folic acid. It can be seen that the effect of the rs1801131 A allele was reduced to a certain extent when patients carried the rs1801133C allele. Each polymorphism site is not independent of the occurrence and development of the disease; there is still some correlation and interaction between the various sites[30]. The construction of the haplotype is the embodiment of genetic association, which can reveal the correlation between multiple SNP and diseases[31].

In this study, MDR software was used to analyse the gene–gene interaction and gene–environment interaction. Our results suggest that the effects of the combination of rs1801133 and rs1801394 on the efficacy of folic acid in the treatment of HHcy were the most stable. It is also true that gene–gene interactions play a role in the treatment effect of folic acid, and the risk of interaction is higher than the risk conferred by single genes. Hcy metabolism involves multiple factors and multiple metabolic pathways. The Hcy level is not only affected by the level of folate but is also related to lifestyle, nutritional factors and genetic factors[32]. For these reasons, the effect of folic acid therapy on HHcy may be affected by gene–gene and gene–environment interactions. Our results show that the risk of treatment failure in the high-risk populations with rs1801133, history of CHD and history of hypertension may be 6·88 times higher than that of the low-risk group (OR = 6·88; 95 % CI 3·98, 16·70). In addition, the three-factor interaction model was the best combination model. Therefore, it is helpful to improve the efficacy of folate therapy by controlling blood pressure at the normal level and actively treating CHD. However, the results were only statistically significant. Further studies are needed to reveal how gene–environment interactions affect the therapeutic effect.

This study aimed to resolve some of the issues found in disease prevention, and it has theoretical and practical value. The high rate of failure of oral folic acid intervention in HHcy has been neglected, and there is a lack of research on the failure of oral folic acid intervention for HHcy. No studies have shown whether the plasma Hcy of patients with HHcy was reduced to normal after folate intervention. This prospective study was carried out to explore the reasons for the failure of folic acid intervention in combination with genetic and environmental factors. In addition, this study had strict quality control in the selection of subjects, data collection and laboratory testing. A questionnaire survey was used to collect the data of the subjects face to face, and all the investigators were trained to reduce the bias.

Nevertheless, there are still some limitations to this study. The plasma folate level of the study group was not detected, and some interactions between folate level and other factors could have influence its efficacy. We did not collect information about other vitamins (B_6 and B_{12}), that may be involved in the metabolic pathway affected by the intervention. In addition, Hcy metabolism involves multiple factors and multiple metabolic pathways, and we only analysed the associations between the gene polymorphisms of some key enzymes in the folate metabolism pathway and the efficacy of folic acid on HHcy, not the gene polymorphisms in other pathways. We also failed to explore the associations of gene–gene and gene–environment interactions in different pathways with the efficacy of folic acid on HHcy.

Conclusion

Folate supplementation can effectively decrease Hcy levels. However, almost half of HHcy patients failed to reach the normal range. *MTHFR* rs1801133 and rs1801131 and *MTRR* rs1801394 and rs162036 were associated with the efficacy of folic acid treatment on HHcy. *MTHFR* rs1801133-rs1801131 haplotype and *MTRR* rs1801394-rs162036 haplotype were associated with the efficacy of folic acid treatment on HHcy. There was a significant interaction between rs1801133 and rs1801394 in the efficacy of folate therapy for HHcy. An interaction between rs1801133, CHD and hypertension was also found. A combination of SNP may be more effective in predicting the risk of failure of folate to alleviate HHcy.

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The authors declare that there are no conflicts of interest.

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