Original Article
A study on the correlation between MTHFR promoter methylation and diabetic nephropathy

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Abstract: Objective: In order to observe the relationship between MTHFR promoter and DN, the determinations on MTHFR promoter methylation level and expression of HCY from DN patients have been carried out. Methods: According to the Diabetes diagnosis and classification standard from WHO in 1999, 85 patients with DM diagnosed by Endocrinology and 30 healthy participants from our medical examination center were chosen as control specimen to study in this paper. All this specimen were divided into A, B, C and D four groups , which are corresponding simple diabetes mellitus group (SDM), early diabetic nephropathy group (EDN), clinical diabetic nephropathy group (CDN) and normal control group. And then, all common materials and clinical experiments data have been collected respectively. (1) Extracted the peripheral blood DNA of each group and determinate the methylation status of MTHFR gene promoter by PCR (MSP). (2) Determinated the serum HCY protein expression of each group. Results: (1) The MTHFR promoter methylation of SDM and diabetic nephropathy group are wear off compared with normal control group. And MTHFR promoter was in demethylation state in normal control group, a slightly weak in SDN, a obviously weak in early diabetic nephropathy group; the MTHFR promoter was in methylation state in clinical diabetic nephropathy group. (2) The HCY protein of simple diabetes mellitus group, early diabetic nephropathy group and clinical diabetic nephropathy group are Pitch with normal control group. HCY protein level of each group are as 7.41±1.61 umol/L, 10.34±2.89 umol/L, 10.95±5.89 umol/L and 13.03±6.14 umol/L corresponding normal control group, simple diabetes mellitus group, early diabetic nephropathy group and clinical diabetic nephropathy group. And there is no statistical significance about the differences among four groups. Conclusion: The demethylation state of MTHFR promoter was obviously weaker in clinical diabetic nephropathy group than in SDM. The level of serum HCY was obviously higher in clinical diabetic nephropathy group than in SDM. It suggested that MTHFR promoter demethylation may be invooed in the pathogenesis of DN.

Keywords: Diabetes meilltus, diabetic nephropathy, methylenetetrahydrofolate reductase, DNA methylation, homocysteine

Introduction

DNA methylation has been an important modification pathway of epigenetics discovered in recent years. The study on the methylation of DNA is the best way to understand the change of epigenetic diseases, as well as an important hot spot for the epigenetic studies.

As a complication in the late stage of diabetes, Diabetic Nephropathy (DN) is a complicated polygenetic disease that occurs in about 40% of diabetic patients. The genome-wide association study (GWAS) has identified that there are differences between African-Americans and Hispanic American Diabetes patients with chronic renal failure and non-associated nephropathy in the methylation of 187 genes [1]. Studies also show that DNA methylation has an influence on the development of type IIII diabetes, and that diabetes is also one candidate disease of DNA methylation [2]. The Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in homocysteine (HCY) metabolism as well as a key catalyzing enzyme in methylation (a reaction that provides one-carbon radical for DNA methylation). The MTHFR promoter region methylation shall affect the activity of MTHFR, resulting in the metabolic blocking of HCY and abnormal methylation. The MTHFR promoter region methylation also participates
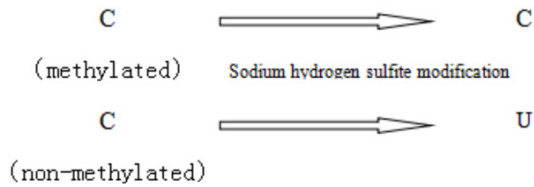
Figure 1. Modification principles of sodium hydrogen sulfite.

in the occurrence and development of the terminal stage of nephropathy [3].

In order to have a further discussion on the effect of MTHFR promoter methylation during the pathogenesis of DN and its possible regulatory mechanism of epigenetics, we detected the overall DNA methylation level of a total genome of peripheral white blood cells of DN patients, and used methylation specificity PCR to detect the state of MTHFR promoter methylation. The purpose of this study is to explain the effect of MTHFR promoter methylation during the DN pathogenesis, so as to provide a new theoretical foundation for the occurrence and development of DN, and to find new ideas for DN's early prevention and treatment.

Experiment method

Objects of study

Inclusion criteria and grouping

Select 85 cases of type II diabetes randomly (37 male cases, 48 female cases) at the endocrinology department of Dalian University Affiliated Zhongshan Hospital from December 2013 to April 2014. The average age of the patients is (60.1±8.41), and all the patients belong to the Han Ethnic Group in Liaoning Province and they are unrelated with each other. The selected diabetic patients meet the diagnostic and classification criterion of *WHO Diabetes 1999* (fasting plasma glucose (FPG) ≥7.0 mmol/l or Random fasting glucose≥11.1 mmol/l or 2 h postprandial plasma glucose≥11.1 mmol/l. The people who meet one of the above standards for two consecutive days shall be diagnosed as DN patients). According to a urine albumin excretion ratio (UAER), patients are divided into: 24 cases of simple diabetes group (B group): UAER<20 ug/min; 34 cases of early Diabetic Nephropathy group (C group): UAER<20-200 ug/min; 27

cases of clinical Diabetic Nephropathy group (D group): UAER>200 ug/min. 30 healthy controls without other diseases and diabetes are selected in the hospital, including 17 female cases and 13 male cases, with an average age of (59.5±6.9). There is no significant age or gender difference between the experiment groups and the control group. The study is approved by the Ethics Committee of Colleges and all of patients signed the informed consent form.

Exclusion criteria

① Certain diseases such as psoriasis, pernicious anemia, malignant tumor, hypothyroidism, systemic lupus erythematosus, hepatopathy, renal dysfunction etc. ② Medicines such as isoniazid, cyclosporine, carbamazepine, levodopa, azar liping, thiazide diuretic, methotrexate, phenytoin sodium, etc. ③ Others: heart failure, primary hypertension, urinary tract infection, chronic nephritis, the exclusion of other endocrine disorders, organ transplantation, etc. Also the people take nonephrotoxic drugs without any occurrence of acute complications.
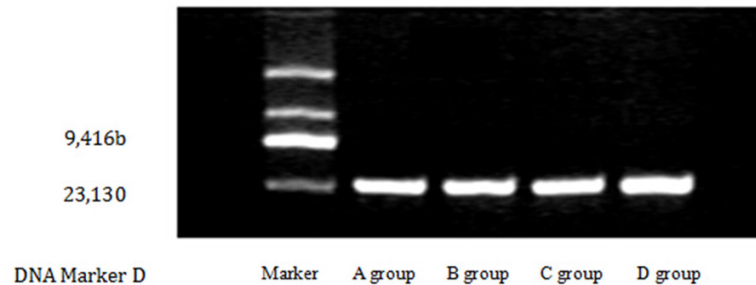
Experiment method

Extracting DNA from the whole blood

Take 200 ul blood and conduct the centrifugation, then put the solution and flocks into an adsorption column CB3 after centrifugation. Collect liquid and pour out the discard solution. Use an ultraviolet spectrophotometer to test the purity and concentration of DNA, Store DNA at -80°C.

Verify the purity of the extracted DNA: Use an ultraviolet spectrophotometer to calculate OD value and DNA concentration: read A260 and A280 optical densities, and the value of A260/A280 shall be in the range of 1.8 to 2.0. DNA concentration (ug/ul)=A260×DNA dilution ratio ×50/1000.

Purification and recovery of DNA and methylation-specific PCR (MSP)

MSP primer design: Use PUBMED to seek complete genome sequences of MTHFR and the specific promoter region sequences. Use study tool software of DNA methylation to seek CPG island and design its primer (**Figure 3**).

Figure 2. Complete genome DNA.

fication system, and see **Table 3** for thereaction condition.

MSP result determination: Take 10 ul MSP product, and undergo 1.5% agarose gel electrophoresis (AGE), under 80 voltages for 40 minutes. Dye with 2 ul EB, and take 50 bp DNA ladder as the marker for sweeping and imaging. Repeat the experiment for 3 times.

Principles and steps of sodium hydrogen sulfite modification. As shown in **Figure 1** *Steps*: Adopt EZ DNA Methylation-GOLD tm KIT (ZYMO Company) to process the DNA of peripheral white blood cells. The operations are as follows: Take 130 ul configured CT Conversion Reagent and 20 ul DNA sample into the adsorption column and centrifuge with full speed. Pour out the discarded solution and add 100 ul M-Wash Buffer, 200 ul M-Desulphonation Buffer into the adsorption column. After centrifugation at full speed, the modified DNA is stored at -80°C.

DNA purification and recovery: Cut agarose gel containing target DNA under UV lamp (ultraviolet lamp), calculate the weight of the gel (record the weight of 1.5 ml centrifuge tube in advance), and take the weight as a gel volume (such as 100 mg=100 ul volume). Add 3 gel volumes of Buffer DE-A and mix well, then heat under 75°C. Discontinuously mixing (every 2-3 min) until the monolithic gel is completely melted. Add Buffer DE-B (half volume of Buffer DE-A) with uniform mixing, and add one gel volume of isopropanol. Suck the mixed liquor of Buffer DE-B, then transfer it to the DNA preparation pipeand conduct 12,000×g centrifugation for 1 min. Pour out the filtrate. Add 500 ul Buffer W 1 and conduct 12,000×g centrifugation for 30 s. Add 700 ul Buffer W 2 and conduct 12,000×g centrifugation for 30 s. Put the preparation pipe into a clean 1.5 ml centrifuge tube, and then add 25-30 ul Eluent into the central of the preparing membrane. Let it rest for 1 min at room temperature. Conduct 12,000×g contrifugation for 1 min and elute DNA.

PCR: The primer was synthesized by Beijing Aoke Ding Sheng Biological Technology Co., Ltd. See **Table 1** for more details: Adopt hot start Taq DNA polymerase, see **Table 2** for the ampli-

Statistical method

SPSS 21.0 is used as the statistical analysis software, and the measurement data shall use $\overline{x}\pm s$ to express. *t-test* is adopted for the comparison between each two groups, and variance analysis and rank sum testing are adopted for multi-group comparisons. The Spearman correlation analysis is adopted in bivariate correlation. When P<0.05 difference was statistically significant.

Results

The general situations and biochemical index of each group

The results show that the plasma HCY level of D group is (13.0±6.1) umol/L, and is higher than C group (11.0±5.9) umol/L, B group (10.3±2.9) umol/L or A group (7.4±1.6) umol/L. The differences between the four groups are statistically significant.

Folic acid and VitB12 of D group are lower than in the control group (A group). Compared with A group, the glycosylated hemoglobin, fasting blood-glucose, triglyceride, diastolic pressure and systolic pressure of other three groups (Group B, C and D) are all increased (**Table 5**).

Complete genome DNA extraction

Four groups' stripes can be watched in the extracted DNA with agarose gel electrophoresis, and the OD value can be tested by using a UV spectrophotometer. The average value of A260/A280 is between 1.8 and 2.0, and results show that there is no protein or RNA contamination (**Figure 2**).
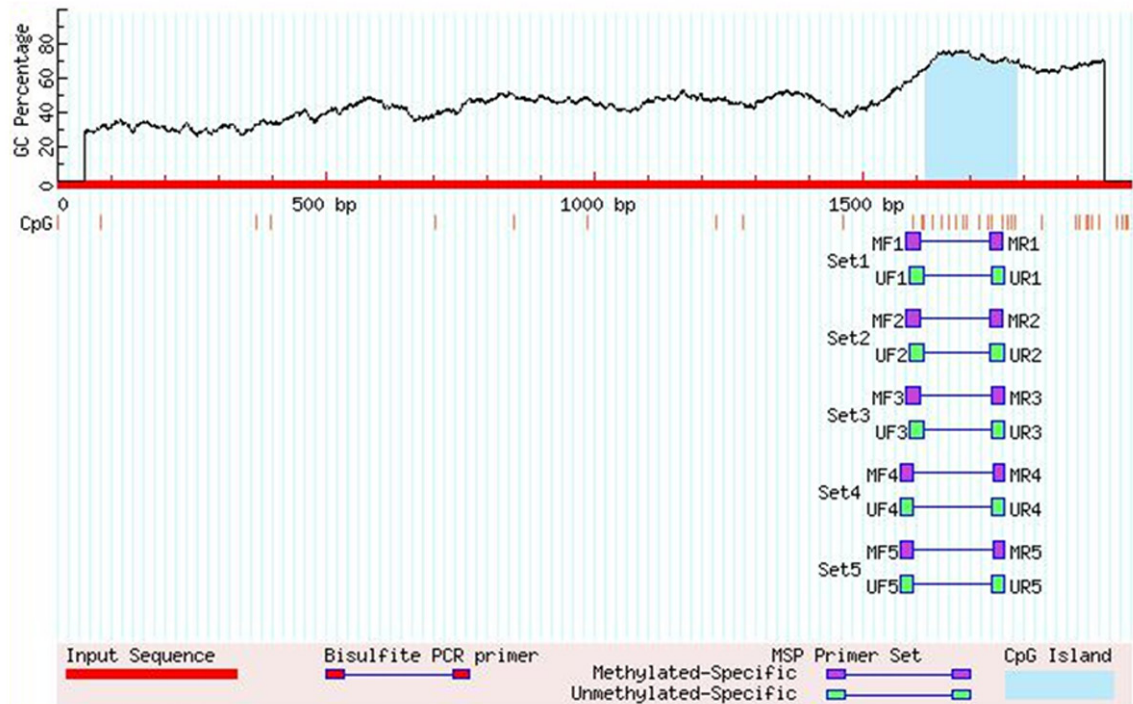
Figure 3. MTHFR gene CPG island analysis results.

Table 1. Primer sequence and product length

Primer name	Primer sequence	Product length
MTHFR (M)	Upstream primer 5'-TAGATTTAGGTACGTGAAGTAGGGTAGAC-3'	170 bp
	Downstream primer 5'-GAAAAACTAATAAAAAACCGACGAA-3'	170 bp
MTHFR (U)	Upstream primer 5'-TTTAGGTATGTGAAGTAGGGTAGATGT-3'	170 bp
	Downstream primer 5'-CAAAAAACTAATAAAAAACCAACAAA-3'	170 bp

Table 2. Amplification System

Reagent	Volume
DNA template after modification of sodium hydrogen sulfite	2.5 ul
dNTP	0.5 ul
PCR buffer	2.5 ul
$MgCl_2$	2 ul
Upstream primer M1 (or U1)	1 ul
Downstream primer M2 (or U2)	1 ul
Hot start Taq DNA polymerase	0.25 ul
Add nuclease-free H_2O till final volume	25 ul

CPG island analysis result of MTHFR

A CPG island was found, the overall length of the CpGis 171 bp, (1618-1788). Percent CG=64.0, ObsExp=1.0 (**Figure 3**).

MSP electrophoresis results of each group

The MSP results show that the methylation levels decrease significantly from A group to D group, and the non-methylation levels increase significantly. A group shows methylation status while D group shows non-methylation status (**Figure 4**).

The occurrence rate of M and U of each group

The occurrence rate of M and U of each group showed the tested F value is 39.513 and 33.317, and the tested probability of *P* value are 0.000, which is less than the significance level (0.05). Therefore, we can assume that the

Table 3. Reaction condition

Temperature	Time	Cycle
94°C	5 min	
94°C	40 s	
64/62°C	40 s	40 cycles
72°C	40 s	
72°C	5 min	

Table 4. Result determination

Result	Strip
Methylation	M
Non methylation	U
Hemimethylation	M+U

occurrence rate of M and U in each group has significant differences. (**Tables 4**, **6** and **Figure 5**).

Spearman correlation analysis of (Homocysteine) and other variables

The spearman correlation analysis on patients' general data, such as plasma HCY level and age, course of disease, BMI, systolic pressure, diastolic pressure, fasting blood-glucose, cholesterol, triglycerides, folic acid, VitB12, MAlb/Cr, demethylation of MTHFR gene, shows that: HCY has a positive correlation with the demethylation of MTHFR gene; and have a negative correlation with folic acid and VitB12; and have positive correlations with BMI, fasting blood-glucose, CHO, and MAlb/Cr, etc. (**Table 7**).

Discussions

Type II diabetes is a kind of polygenetic disease. The polygenic diabetes accounts for 95% of total type II diabetes. Nearly 400 candidate genes for type II diabetes are revealed by the genome-wide association study (GWAS) and meta-analysis [4]. The occurrence of type II diabetes might relate to many factors, such as heredity, environment, diet, etc. Many genes participate in the development of type II diabetes and its complications, and each gene has different effects and interactions with each other. The modification of DNA methylation and histone can be changed with the environment, and different disease-causing susceptible genes may affect different metabolic links of diseases. It reminds us that the diabetic patients have not only abnormal genetic struc-

tures, but also defects in genetic expression and modification after the expression [5]. These then participate in the occurrence and development of type II diabetes. DN (Diabetic Nephropathy) is one of the serious complications of diabetes, easily leading to end-stage renal disease. The exact pathogenesis of DN has not been elucidated completely, and the data of clinical observation and epidemiology shows that genetic predisposition is an important factor in the occurrence of DN. In recent years, researchers have performed in-depth studies on the epidemiology, and genome sequence variation to explore the occurrence mechanism of the disease by other factors. It may have a more important significance on correctly understanding the diseases associated with external factors.

DNA methylation is an important molecular mechanism of epigenetics, causing changes in chromatin structure, DNA conformation, DNA stability and the interaction between DNA and protein. Being able to control gene expression, it thus is an important gene regulation method [6]. DNA methylation is a reaction under the effect of DNA methyl transferase, the reaction will take s-adenosylmethionine as methyl donor to catalyze the transfer of cytosine to 5-methyl-cystein. This modification reaction mainly occurs in the cytosine of CPG island dinucleotide. In previous studies, it was generally believed that DNA hypermethylationis correlated with gene silencing, and hypomethylation or demethylation is correlated with gene activation. Some studies show that there is some DNA hypermethylation of CPG rich region within genes which have a positive correlation with gene expression. Apossible mechanism is: through controlling the transcription of antisense RNA or micro RNA in the gene, its down regulation of host gene expression could be released [7]. The gene expression is not only effected by gene mutation, but also effected by DNA methylation; the change of DNA methylation (i.e., normal methylation or the aberrant methylation of non-methylated gene sequence)-will lead to the occurrence of diseases [8].

There are no TATA boxes in the MTHFR promoter region, but it has CPG island and many potential Sp1 binding sites, which provide the structure foundation of methylation-regulation for MTHFR gene expression. The study of cul-

Table 5. Each general situation and biochemical index test results

Group	The number of cases (Male/Female)	Age (age)	Smoking (%)	Course of disease (year)	BMI	Systolic pressure (mmHg)	Diastolic blood pressure (mmHg)	Cholesterol (mmol/L)
A group	30 (13/17)	51±9	10.0		25.0±2.5	75±5	126±9	4.3±0.6
B group	24 (11/13)	52±10	15.0	9±5	25.0±2.6	79±6☆	138±10☆	4.4±0.8
C group	34 (14/20)	52±12	24.3	9±6	23.3±3.3	82±7☆,Δ	139±12☆,Δ	4.7±0.8
D group	27 (12/15)	57±10	21.4	11±5	24.2±2.9	85±7Δ,☆,Φ	139±15Δ,☆,Φ	4.9±0.9
F value	0.917*	1.410#	2.979#	32.562	2.180	11.203	6.853	4.955
P value	0.775	0.244	0.222	0.000	0.094	0.000	0.000	0.008

Group	Triglyceride (mmol/L)	Fasting blood glucose (mmol/L)	HbA1c (%)	HCY (umol/L)	Folic acid (mg/L)	VitB12 (ng/L)
A group	1.6±0.6	3.8±0.5	4.5±0.7	7.4±1.6	12.8±3.4	1079±575
B group	1.9±1.0☆	8.7±2.9☆	7.8±2.7☆	10.3±2.9☆	10.8±3.4☆	1083±667☆
C group	2.1±1.2☆,Δ	9.8±3.3☆,Δ	8.4±2.2☆,Δ	11.0±5.9☆,Δ	8.4±3.7☆,Δ	1304±668☆,Δ
D group	2.2±1.2☆☆,Φ	10.9±4.9☆☆,Φ	8.8±1.8☆☆,Φ	13.0±6.1☆☆,Φ	7.5±4.4☆☆,Φ	1627±564☆☆,Φ
F value	1.782#	1.403#	27.848#	7.118#	3.35#	4.676#
P value	0.010	0.001	0.010	0.000	0.018	0.004

Note: BMI, Body mass index; HbA1c; Glycosylated hemoglobin; HCY Homocysteine levels; vitamin B12, vitamin B12; *, χ^2 value; #, Rank sum test results; ☆ and A group compared; Δ and group B compared; Φ and group C compared, $P<0.05$.
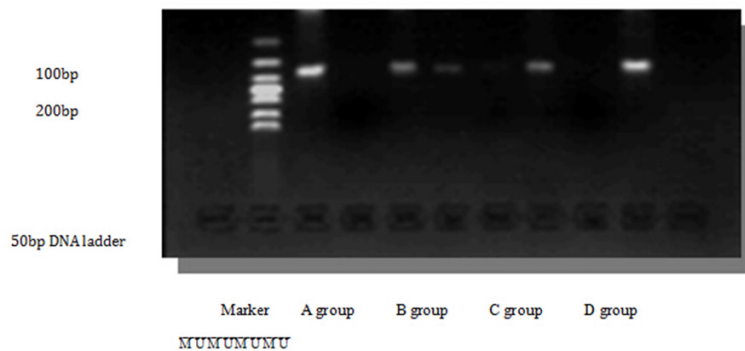
Figure 4. Methylation of MTHFR gene promoter results of each group. Note: A group: Control group; B group: Simple diabetic group; C group: Early Diabetic Nephropathy group; D group: Clinical Diabetic Nephropathy group. M: methylation primer amplified fragments; U: non-methylation primer amplified fragments.

**Table 6.** Each group M and U Emergence rate

Group	M		U	
	Mean value	Standard deviation	Mean value	Standard deviation
A group	93.33%	25.37%	0.00%	0.00%
B group	66.67%☆,Φ	48.45%☆,Φ	41.67%☆,Φ	50.36%☆,Φ
C group	26.47%☆,Δ	44.78%☆,Δ	73.53%☆,Δ	44.78%☆,Δ
D group	0.00%☆,Δ,Φ	0.00%☆,Δ,Φ	88.89%☆,Δ,Φ	32.03%☆,Δ,Φ
F value	39.51		33.32	
P value	0.000		0.000	

☆ and A group compared; Δ and group B compared; Φ and group C compared, P<0.05. Note: P<0.05.

MTHFR gene promoter region exhibits demethylation, and that the MTHFR mRNA expression was increased. The high HCY may cause DNA hypomethylation of the genome, thus inducing some kinds of rheumatism immune diseases. The experiments of end-stage renal disease in Egyptian patients show that [9] the MTHFR methylation may become one mechanism of high HCY of organisms in chronic end-stage renal disease patients, and may play a certain role in the disease pathogenesis [3]. The rate of MTHFR promoter methylation is 3 times as great as in normal groups, and the MTHFR methylation of end stage renal disease patients is higher than in the normal control group, suggesting the influence of MTHFR gene epigenetics silencing on the development of chronic end-stage renal disease. However, different from the conclusion above, the Spearman correlation analysis of the experiment shows that when compared with the normal control group, the Methylation of MTHFR gene promoter of

ture in vitro vascular smooth muscle cells for HCY at different concentrations shows that its
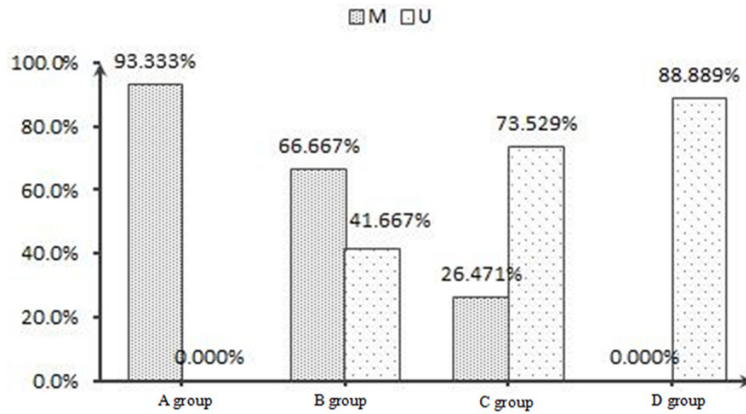
Figure 5. Occurrence rate of M and U of each group.

Table 7. Homocysteine levels and other variable spearman correlation analysis

Project	Correlation coefficient	P value
Age	0.119	0.206
Course of disease	0.150	0.017
BMI	0.214	0.000
Systolic pressure	0.032	0.615
Diastolic blood pressure	0.031	0.625
Fasting blood glucose	0.041	0.025
TG	0.097	0.124
CHO	0.171	0.007
Folic acid	-0.239	0.010
VitB12	-0.338	0.000
MAlb/Cr	0.310	0.000
MTHFR	0.186	0.016

cess of 5,10-methylenetet-raydrofolate into 5-methyle-netetraydrofolate, thereby affecting the methionine circulation, inducing HCY aggregation, and playing a part in the occurrence and development of DN. Moreover, the HCY aggregation will affect the methionine circulation, control the transferring of methylation and then control the methylation, making a vicious circle as a result. Khazamipour *et al.* [10] have made a study on MTHFR gene promoter region methylation of non-obstructive azoospermia, and the result shows that there is no abnormal methylation of MTHFR gene promoter region in the bold preparation, but there is abnormal methylation inpatients' testicular tissue. Moreover, there is non-methylation in the control group, but the supermethylation was only found in some special tissues, suggesting that there is disease particularity and gene specificity in abnormal methylation [11]. Therefore, we shall not only consider the correlation between DN and methylation of MTHFR gene promoter, but also consider the disease particularity and gene specificity.

The experiment has certain limitations, such as the small volume of sample, and the absence of part of renal tissue's methylation. If the comparison between the methylation situation of renal tissue and peripheral blood is made, it can better explain the abnormal methylation of MTHFR gene promoter better, as well ascomprehensively explaining the effects of methylation of MTHFR gene promoter on DN. High HCY may influence the multiplication of vascular smooth muscle cells, direct cytotoxicity and blood coagulation system and then cause vasculopathy. In addition, High Hcy may control the expression of nitric oxide synthetase, so as to reduce the compound of nitric oxide, damage the stability of vasoconstrictor and vasodilator, and become a risk factor for Diabetic Nephropathy (DN). Many experiments have confirmed that the increase of HCY is an independent risk factor of DN [12]. According to our study, serum HCY has an increased tendency with the development of DN, and the HCY levels of each group have statistical differences, suggesting a correlation between HCY and DN development. Although there are no significant

patients in the simple diabetes group, early Diabetic Nephropathy group and clinical Diabetic Nephropathy group demonstrate a weakening trend, and fragment occurrence rate of clinical diabetic group amplified in the non-methylation primers of MTHFR gene is 88.89%. Theserum HCY expression showed a gradually increased tendency, suggesting that HCY expression is positively correlated with MTHFR gene promoter demethylation. The change of methylation of MTHFR gene promoter is a key reason for the down regulation of MTHFR expression, which may affect the metabolism of HCY. Therefore, the MTHFR gene promoter demethylation is one possible reason for DN morbidity. This specific mechanism is not clear yet, We speculate that the MTHFR gene promoter demethylation may change the activity of MTHFR, and block the transfer pro-

differences of total cholesterol in each group, it shows a positive correlation between HCY level and serum total cholesterol in each group. The possible mechanism is like this: the gene expression activates the HMG-CoA degrading enzyme and stimulates the generation of cholesterol. The reasons may be caused by the participation of some other factors such as nutritional status, chronic inflammation, etc. The conclusion is also consistent with many past studies [13].

The MTHFR gene expression is changed by high HCY through changes in the DNA methylation modification. Its key enzyme shall be induced and catalyzed by HCY by transferring into methionine, and MTHFR gene demethylation and mRNA's high expression is obviously conducive to decrease the abnormally high HCY. This is a compensatory mechanism instead of a damage effect of high HCY, suggesting that though the environmental factor has a damaging or pathogenic effect on gene methylation modification, it may also be a compensatory reaction. The DNA modification of epigenetics has a potential reversibility, which provides a powerful proof for future genome treatments and may prevent the deterioration of renal function.

Although it has been discovered that many "candidate genes" and chromosome locus are related with Diabetic Nephropathy, most correlations are not verified in different kinds of races and diseases, and their functions during the process of disease are not clarified. As a polygenetic disease, it would not have a single testing method for DN in the future. Therefore, it istheoretically and practically significant to clarify why some genes appear demethylation and someothers appear gene hypermethylatiom under the influence of the same factor, as well as to clarify which DNA methylation modifications are pathogenic and which are compensatory. The study of genes plays a significant role in providing the specific pathway of DN pathogenesis and explaining the DN physio-pathologic mechanism, which may help develop the new strategies of early diagnosis, prevention and treatment, and reduce the morbidity and case fatality rate of DN.

Disclosure of conflict of interest

None.

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