ORIGINAL ARTICLE

Effect of plasma homocysteine levels on methylation and expression of thrombomodulin gene in patients with retinal vein occlusion

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ABSTRACT

Objective: To explore the effect of plasma homocysteine (Hcy) levels on methylation and expression of thrombomodulin (TM) gene in patients with retinal vein occlusion (RVO).
Methods: 11 cases of patients who were diagnosed as RVO in Department of Ophthalmology in Baogang Hospital from January 2019 to December 2020 were included in this research. 11 cases of healthy people who came to our physical examination center for physical examination during the same period were included into the control group. Fasting cubital venous blood was collected in the morning, plasma Hcy levels were measured by means of enzyme cycle assay, TM gene methylation was detected by methylation-specificity polymerase chain reaction (MSP), and the correlation between characteristic changes in plasma Hcy and TM gene methylation was analyzed in the research subjects.
Results: Plasma Hcy expression levels were higher in the RVO group than those in the control group ($p < .01$). In the control group, the TM primer set region was basically unmethylated, while in the RVO group, the TM primer set region was partially methylated.
Conclusions: Plasma Hcy affects the occurrence and development of RVO through methylation of TM encoding gene.

Key Words: Retinal vein occlusion, Homocysteine, Thrombomodulin, Methylation

1. INTRODUCTION

Retinal vein occlusion (RVO) is a common retinal vascular disease that usually results from thrombosis or compression inside the retinal veins. The disease mainly affects the middle-aged and elderly population and gradually increases with age. An data analysis from all hospital-treated RVO patients in UK between 2000 and 2015 showed that the proportion of Branch Retinal Vein Occlusion (BRVO) increased from 60% to 70%, and the proportion of Central Retinal Vein Occlusion (CRVO) decreased from 40% to 30%.[1] In mainland China, according to the data published at the "Beijing Fundus" Forum held by Beijing Ophthalmology Association in 2018, retinal vein occlusion has become one of the second leading blinding eye diseases in China, and the number of new cases each year is gradually increasing.[2] At present, it is generally believed that the occurrence of RVO is related to various factors such as vascular endothelial cell injury, abnormal hemorheology, and coagulation system disorders and other factors.

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Homocysteine (Hcy) is a very important type of thioamino acid and has a variety of biological functions in human body. Studies have shown that Hcy is involved in the process of protein synthesis and DNA repair; it has an effect on cardiovascular diseases such as arteriosclerosis and coronary heart disease by affecting important indicators such as lipoproteins;[3] in hepatocytes, Hcy can also promote glutamyl transferase activity and increase the aspartate metastasis rate in hepatocytes.[4] In recent years, an increasing number of studies have shown a close relationship between Hcy and RVO. Hcy may contribute to RVO by inducing endothelial cell apoptosis, promoting atherosclerotic plaque formation, and increasing blood viscosity.[5,6] Thrombomodulin (TM) is an important anticoagulant molecule on endothelial cells, and the high level of Hcy can lead to abnormal DNA methylation modification at CpG (Cytosine-phosphate-Guanine) island sites in the TM promoter region, which in turn regulates the expression of the gene encoding TM.[7] Laboratory models have also demonstrated that abnormal DNA methylation modification significantly reduces TM mRNA and protein levels in endothelial cells.[8,9]

However, the specific mechanism of action of Hcy on RVO has not been clearly studied. Therefore, the purpose of this study was to investigate the effect of Hcy levels in plasma on the methylation and expression of genes encoding thrombomodulin in RVO.

2. Objects, materials and methods

2.1 Subjects

1. RVO Group: 11 patients diagnosed with RVO from January 2019 to December 2020 in the Department of Ophthalmology of Baogang Hospital, were randomly selected, all of whom were Han Chinese and unrelated to each other. Inclusion criteria: (1) Excluding the effect of diet or lifestyle habits on plasma Hcy, all study subjects lived locally for a long time lifestyle habits on plasma Hcy; (2) All patients with RVO had a sudden onset and were able to clearly describe when visual acuity decreased or visual field defects developed; (3) patients above 45 years old. Exclusion criteria: (1) Patients with RVO complicated with central retinal artery occlusion or branch retinal artery occlusion were excluded; (2) Patients with other ocular diseases were excluded, such as diabetic retinopathy, glaucoma, uveitis, etc.; (3) Patients with severe systemic disease, vasculitis, renal, hepatic, thyroid or cardiovascular diseases were excluded. 2. Control group: 11 healthy people who came to our physical examination center for physical examinations were selected. The experiment was conducted in conformed to the Declaration of Helsinki and had been approved by the Ethics Committee of Baogang Hospital, and all subjects had signed informed consent.

2.2 Materials and instruments

Taq Plus DNA polymerase, 10× PCR Buffer (containing Mg2 +) and primers were purchased from Sangon Biotech; 6× DNA Loading Dye and DNA Ladder Mix (100-10000 bp) were purchased from ThermoFisher (USA); PCR instrument was purchased from ABI (USA); gel imager was purchased from Shanghai Furi Technology Co., Ltd.; electrophoresis instrument was purchased from Beijing Liuyi Instrument Factory; micro-vortex mixer was purchased from Shanghai Huxi Analytical Instrument Factory Co., Ltd.; Merinton SMA4000 UV-visible microspectrophotometer was purchased from Merinton (Beijing) Instrument, Ltd.

2.3 Study methods

2.3.1 *Specimen collection and storage*

A total of 5 ml of fasting cubital venous blood was collected in the morning from the RVO group and the control group, stored with a numbered anticoagulant tube and frozen in a -80°C cryogenic refrigerator for future use. Blood samples were numbered: 1 N-1; 2 T-1; 3 N-2; 4 T-2; 5 N-3; 6 T-3; 7 N-4; 8 T-4; 9 N-5; 10 T-5; 11 N-6; 12 T-6; 13 N-7; 14 T-7; 15 N-8; 16 T-8; 17 N-9; 18 T-9; 19 N-10; 20 T-10; 21 N-11; 22 T-11. N stands for the control group and T represents the RVO group.

2.3.2 *Hcy analysis*

Plasma Hcy levels were measured using an enzyme cycle assay and analyzed by technicians from the clinical laboratory of Baogang Hospital.

2.3.3 *MSP detection of TM gene methylation*

Methylation-specificity polymerase chain reaction (MSP) was used to detect gene methylation in this experiment. The basic principle is as follows: the genomic DNA is treated with sodium bisulfite, unmethylated cytosines are changed into uracil, while methylated cytosines are unchanged, and then the same nucleotide sequence of the tested gene is amplified with two pairs of specific primers. The amplified products are analyzed by DNA agarose gel electrophoresis and gel scanning.

(1) DNA extraction and quality inspection

TM gene DNA extraction was performed using the Ezup Column Animal Tissue Genomic DNA Drawer Kit (Sangon Biotech) according to the reagent instructions. The experimental results were detected using a Merinton SMA4000 UV-Vis microspectrophotometer.

(2) Primer design

M indicates methylated primer and U stands for unmethylated primer. Upstream primer MPA116-1MF: GTT CGC GTT AGG GGA GGC; Downstream primer MPA116-1MR: TAC GAC AAA CAC GCA CCG ATA; Upstream primer

MPA116-1UF: GGG TTT GTG TTA GGG GAG GT; Downstream primer MPA116-1UR: TAC AAC AAA CAC ACA CCA ATA AAA.

(3) PCR reaction system
Template DNA 20-50 ng/μl, 2 μl; Primer F 10 μm, 2 μl; Primer R 10 μm, 2 μl; dNTP (mix) 10 μm, 3 μl; Taq Buffer (with $MgCl_2$) 10 $\times$ 5 μl; Taq 5 U/μl, 0.5 μl; with an addition of ddH_2O to 50 μl.

(4) PCR reaction conditions
The PCR reaction conditions were as follows: Predenaturation at 95°C for 3-5 min, denaturation at 94°C for 30 sec, annealing at 55-60°C for 25-30 sec, extension at 72°C for 30-50 sec, 20 cycles, and repair and extension at 72°C for 5-8 min.

(5) Detection of TM gene methylation
PCR products were electrophoresed on 5 μl of 1% agarose gel at 150 V, 100 mA, for 10-20 min, and the results were observed and analyzed by gel scanning.

2.4 Statistical methods
SPSS 26.0 was used for statistical analysis, and quantitative data were represented by Mean $\pm$ SD, and the difference was statistically significant ($p < .05$).

3. RESULTS

3.1 Relationship between the expression of plasma Hcy and clinical characteristics in the two groups
There were 6 males and 5 females in the RVO group, with the age of 64.27 $\pm$ 10.09; and there were 5 males and 6 females in the control group, with the age of 63.19 $\pm$ 8.92. The expression level of Hcy in plasma in the RVO group was higher than that in the control group ($p < .01$) (see Table 1).

Table 1. Relationship between the expression of plasma Hcy and clinical characteristics

	RVO Group (n = 11)	Control Group (n = 11)	*p* value
Age	64.27±10.09	63.19±8.92	> .05
Gender			
Male	6(54.5%)	5(45.5%)	> .05
Female	5(45.5%)	6(54.5%)	> .05
Plasma Hcy (μM/L)	16.19±10.65	9.51±4.62	< .01

3.2 DNA marker
Thermo ScientificTM GeneRulerM DNA Ladder Mix was used to determine approximately quantitative ranges of double-stranded DNA on agarose gels. It consists of 21 chromatographically purified DNA fragments: 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100. It contains three reference bands: 3000, 1000 and 500 bp for easy localization (see Figure 1).

3.3 Detection of TM gene methylation
U primers for specimens 1, 2, 7, 8, 10, 19, 21 had a normal amplification, and no amplification with the M primer: the specimen was essentially unmethylated in the primer setup region. U primers for specimens 5, 6, 14, and 20 were amplified normally, and a small amount of M primers were amplified: most specimens were unmethylated in the primer setup region, and a small number of specimens may have a high degree of methylation in the primer setup region. Both U and M primers for specimens 3, 4, 9, 11, 12, 13, 15, 16, 17, 18, 22 had a normal amplification: the specimen primer setup region was partially methylated. According to the brightness of the amplified bands of M primers, it is speculated that the methylation degrees of the samples in the primer setup area are listed as follows: 22 > 3 $\approx$ 9 $\approx$ 12 $\approx$ 13 > 4 $\approx$ 11 $\approx$ 15 $\approx$ 16 $\approx$ 17 $\approx$ 18 > 5 $\approx$ 6 $\approx$ 14 $\approx$ 14 $\approx$ 20 > 1 $\approx$ 2 $\approx$ 7 $\approx$ 8 $\approx$ 10 $\approx$ 19 $\approx$ 21 (see Figure 2).

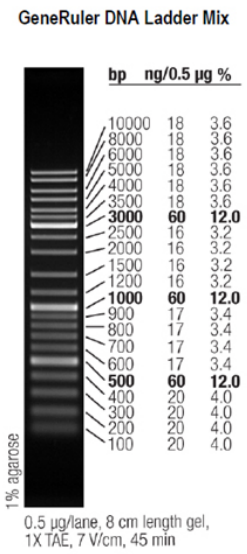

Figure 1. Schema of DNA marker

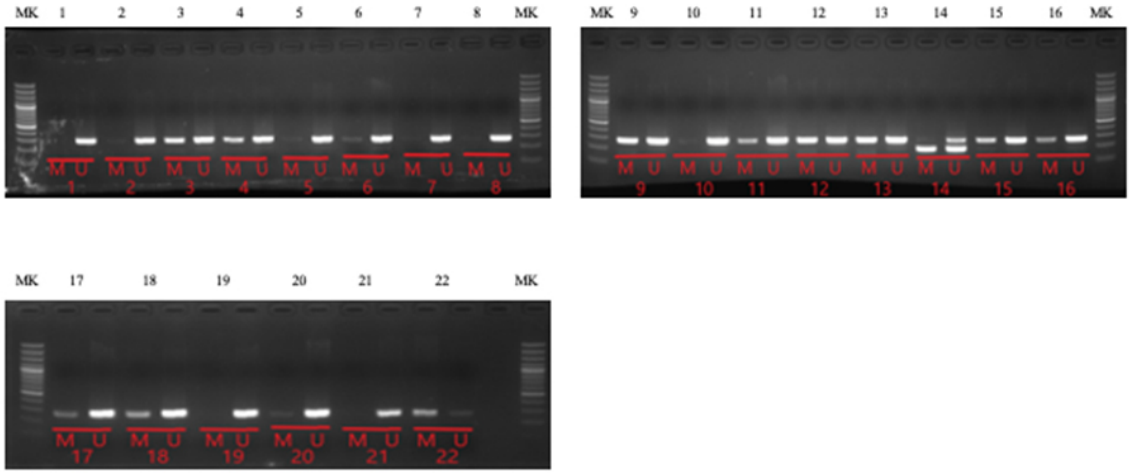

Figure 2. Schema of DNA marker

4. Discussion

RVO is a retinal vascular disease associated with abnormalities in blood coagulation and circulatory system, and its pathogenesis is complex: the presence of fresh or recanalized thrombi in the central retinal vein near the cribriform plate leads to luminal narrowing and increased blood flow;[10] vascular stasis and exudation stimulate the secretion of inflammatory factors, causing focal phlebitis and optic nerve head swelling;[11] ischemia and hypoxia increase oxidative stress response, stimulate the secretion of inflammatory markers and caspase-9;[12] other cardiovascular risk factors such as high cholesterol, high triglycerides, and Hcy may negatively affect microcirculatory function, and further promote local coagulation and aggravate the severity of RVO.[13] Hcy is the most thoroughly investigated risk factor for thrombosis in RVO.[14] Normally, Hcy is transformed to methionine via the methylation metabolic pathway or to cysteine via the transsulfurase metabolic pathway. However, when there is insufficient methyl donor or some gene mutation in the body, the metabolic pathway of Hcy will be changed, resulting in its accumulation in the body. In addition, factors such as smoking, high-fat diet, and lack of vitamins B6, B12, and folic acid also increase Hcy levels.[15] Hcy usually damages the vascular endothelium, causing platelet and lipid aggregation to form thrombi. In addition, Hcy produces free radicals due to oxidative stress, destroys the vascular endothelium and promotes the formation of thrombi, which leads to RVO. In this study, we found that Hcy expression levels in plasma were higher in the RVO group than those in the control group ($p < .01$), which was consistent with a study from Koylu MT et al.[16]

TM is a type I transmembrane glycoprotein, consisting of 557 amino acids and expressed by vascular endothelial cells, can regulate thrombus formation and coagulation responses. Under normal circumstances, TM binds to thromboxane and inhibits thromboxane coagulation activity, thereby preventing thrombosis inside the vessel. In addition, TM can also bind to plasminogen activator complex and promote fibrinolysis, thereby accelerating the degradation of thrombi. In recent years, an increasing number of studies have shown that TM plays an important role in the development and progression of many diseases. For example, abnormal TM expression and dysfunction are closely associated with the development and progression of disease in terms of liver disease, kidney disease, cardiovascular disease, and infection.[17] Abnormal expression of TM in RVO has been shown by Von et al.[18] DNA methylation is a major type of epigenetic regulation affecting disease pathogenesis and occurs as a methyl modification of the fifth carbon of cytosine (5-methylcytosine, 5mC), found in the context of mammalian symmetrical CpG dinucleotides. It can often silence gene expression.[19] Some evidence suggests that Hcy can lead to global DNA hypomethylation and specific gene promoter hypermethylation. TM promoter methylation has been reported to be associated with tumor growth, invasion, and lymph node metastasis and also promotes the development and progression of coronary heart disease.[20] Recent studies have shown that Hcy can promote methylation of TM gene and decrease the expression level of TM by promoting the expression and activity of DNA methyltransferases, thereby affecting thrombosis and coagulation response.[21] In our study, the TM primer setup region was essentially unmethylated in the control group, whereas the TM primer setup region was partially methylated in the RVO group.

5. Conclusion

In summary, the plasma Hcy expression level was increased in the RVO group, and the primer setup region of TM was

partially methylated, and studies have shown that plasma Hcy affects the occurrence and progression of RVO through methylation of the gene encoding TM. In this study, the sample size was small, and the specific mechanism of action of plasma Hcy on TM gene methylation was not investigated, which will be further studied in the above aspects in the future.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare they have no conflicts of interest.

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