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ORIGINAL RESEARCH

Plasma Homocysteine Thiolactone Associated with Risk of Macrovasculopathy in Chinese Patients with Type 2 Diabetes Mellitus

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ABSTRACT

***Introduction*:** This study investigated the role of homocysteine thiolactone (HcyT) in the development of macrovascular complications in Chinese patients with type 2 diabetes. HcyT has been proposed as a possible molecular basis for homocysteine (Hcy)-induced vascular damage.
Methods: One hundred and sixty subjects were recruited into this study: 40 healthy controls and 120 patients with type 2 diabetes. Plasma Hcy levels were measured by polarization immunoassay and HcyT concentrations were monitored using high-performance liquid chromatography on a reverse-phase C18 column with ultraviolet detection. Plasma folic acid and vitamin B_{12} levels were measured using radioimmunoassay methods.
Results: Plasma Hcy and HcyT concentrations in patients with type 2 diabetes were significantly higher than in healthy controls (Hcy [25th and 75th quartiles]: 9.28 [7.51-11.82] vs. 5.64 [5.17-8.00] μmol/L, P=0.01; HcyT: 3.38 [2.94-4.73] vs. 2.91 [2.77-3.08] nmol/L, P<0.05). Plasma Hcy and HcyT levels in patients with macrovasculopathy (MAVP) were significantly

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higher compared with patients without MAVP (Hcy: 10.36 [7.67-12.45] vs. 7.85 [6.76-10.52] μmol/L, $P<0.05$; HcyT: 4.27 [3.02-5.11] vs. 3.12 [2.63-3.77] nmol/L, $P<0.05$). Plasma HcyT concentrations were positively correlated with urinary excretion of albumin/creatinine (Alb/Cr; $r=0.285$, $P=0.007$), duration of diabetes ($r=0.249$, $P=0.019$), age ($r=0.233$, $P=0.028$), and fibrinogen levels ($r=0.289$, $P=0.034$). Plasma HcyT concentrations were negatively correlated with high-density lipoprotein levels ($r=-0.223$, $P=0.037$). Binary logistic regression showed that HcyT, Hcy, smoking, serum triglyceride, and urine Alb/Cr were significantly associated with the risk of diabetic MAVP ($P<0.05$).

Conclusion: Hcy and HcyT levels were associated with the development and progression of diabetic MAVP. HcyT may provide a plausible chemical mechanism for explaining Hcy toxicity in the human vascular endothelium.

Keywords: diabetes; homocysteine; homocysteine thiolactone; vasculopathy

INTRODUCTION

Homocysteine (Hcy) is a sulfydryl amino acid that has been widely accepted as a novel risk marker associated with peripheral, coronary, and cerebral arterial diseases.[1,2] Possible cellular mechanisms by which Hcy may contribute to cardiovascular disease include the unfolded protein response, oxidative stress, and the induction of proinflammatory factors.[3] However, the exact mechanism of Hcy toxicity has still not been confirmed. It has been recently reported that Hcy thiolactone (HcyT)—formed by enzymatic conversion of Hcy in all cell types—is potentially harmful and may be the molecular basis for Hcy-induced vascular damage.[4-6]

Diabetes is a major health problem afflicting millions of people globally.[7] It may be associated with other conditions including macro- or microvascular complications, dyslipidemia, or hypertension. Retrospective and prospective studies have demonstrated that plasma total Hcy is a risk factor for macrovasculopathy (MAVP) in diabetic patients.[8,9] Based on the hypothesis that HcyT could more directly predict the risk of MAVP, the association of HcyT with MAVP was investigated, and the correlations of HcyT with other biochemical variables were analyzed in this study.

MATERIALS AND METHODS

Subjects

One hundred and twenty patients diagnosed with type 2 diabetes who visited the Chinese PLA General Hospital (Beijing, China) and 40 healthy (nondiabetic) controls were enrolled.

Exclusion criteria included patients with glycated hemoglobin (HbA_{1c}) >12%.

Patients with diabetic ketoacidosis, other systemic diseases, severe hepatic or renal diseases (serum creatinine >133 μmol/L), fever, congestive heart failure, as well as acute cardiovascular and cerebrovascular events were also excluded. Patients who received vitamin B complex or folic acid during the past 6 months were also excluded. The study was approved by the ethics committee affiliated to the Chinese PLA General Hospital and written informed consent was obtained from all participants.

The diagnosis of cerebral infarction or bleeding was made by medical history and confirmed by computed tomography of the brain. Coronary heart disease (CHD) was considered when at least one of the following existed: a history of myocardial infarction or angina pectoris; typical ischemic alterations in electrocardiogram (ECG); or previous finding of atherosclerosis from arteriography of the coronary artery. Peripheral vascular disease (PVD) was identified by a finding of lower-limb intermittent claudication and absent foot pulses. Adverse events were recorded at the end of the study.

Laboratory Analysis

Blood samples were drawn from subjects after an overnight fast: 6 mL blood was drawn for the measurement of Hcy, HcyT, folic acid, and vitamin B_{12}; 8 mL blood was drawn for the other parameters. Plasma glucose was measured with a glucose-oxidase method. Serum total cholesterol, triglycerides, high-density lipoprotein (HDL), and other biochemical parameters were measured using an autoanalyzer (HITACH7600, Japan). HbA_{1c} was measured with chromatography (BIO-RAD Variant, USA). Vitamin B_{12} and folate levels in plasma were assessed using a competitive protein binding assay (chemiluminescence assaying kit). Urinary albumin/creatinine (Alb/Cr) was assayed using radioimmunoassay methods (DCA2000, USA).

Plasma Hcy levels were measured by polarization immunoassay and the intra- and inter-assay coefficients of variation were 2.0% and 3.2%, respectively. For HcyT measurement, plasma was collected and chilled on ice. The plasma was separated by centrifugation at 4°C (2000 g) for 15 minutes. The HcyT concentration was measured using high-performance liquid chromatography (HPLC) on a reverse-phase C18 column with ultraviolet detection.[10] Human plasma samples (0.4 mL) were deproteinized by ultrafiltration through Millipore 10-kD cut-off membranes at 4°C (Millipore, USA). The ultrafiltrate was treated with 1 mol/L K_2HPO_4 (10 μL) and chloroform/methanol (2:1 V/V) sequentially to adsorb HcyT.[10] The organo-phase was transferred into another tube and dissolved in 0.1 mol/L hydrochloric acid, followed by centrifugation at 15,000 g for 5 minutes at 4°C. The supernatant was evaporated at −56°C under a nitrogen atmosphere. The dried matter was dissolved in deionized water, and then analyzed by HPLC instrumentation. Samples (up to 20 μL) were injected into a Beckman reverse-phase C18 column equilibrated with 5% acetonitrile, 10 mmol/L K-phosphate buffer, pH 7.4, at 25°C. The column was eluted at

1 mL/minute. The effluent was monitored at A240; the UV absorption maximum of HcyT was ε=3500 mol-1 cm-1. The detection limit was 1 pmol/L HcyT. The intra- and inter-assay coefficients of variation were 2.7% and 5.3%, respectively.

Statistical Analysis

Data were analyzed using the SPSS. Clinical data with normal distribution were expressed as mean±SD, and results with non-normal distribution were shown as the median with 25th and 75th quartiles. Data between the two groups were compared by Student's *t* test/Mann Whitney *U* test. The chi-square test was used to evaluate differences in frequencies. The relationship between plasma HcyT and other biochemical parameters was estimated by Spearman's correlation analysis. Binary logistic regression was used to assess the risk factors for diabetic macrovascular complications. A *P* value of <0.05 was considered significant.

RESULTS

Subjects

Of the 120 patients with type 2 diabetes, 68 patients were treated with oral hypoglycemic agents alone, 24 with insulin alone, and 28 with insulin in combination with oral hypoglycemic agents. Sixty-three patients had MAVP. Twenty-six patients had cerebrovascular disease alone (18 cerebral infarction, eight cerebral bleeding), 26 had CHD (12 myocardial infarction, 10 angina pectoris, four ischemic ECG changes), and 19 had PVD. Three patients with cerebrovascular disease and five with PVD were also complicated with CHD. There were no reported incidences of adverse events.

Table 1 shows the clinical characteristics and laboratory findings in the group of patients with diabetes compared with controls. Between the groups, there were no significant differences in sex, age, body mass index (BMI), and plasma vitamin B_{12} levels. However, the ratio of urinary Alb/Cr in the diabetes group was higher than in the nondiabetes group ($P<0.001$), and serum HDL and folate levels were significantly lower ($P<0.05$).

There were 32 patients with diabetic nephropathy in the MAVP group, and 27 patients with diabetic nephropathy in the non-MAVP group ($P>0.05$). There was no difference in the incidence of diabetic retinopathy between the two groups (29 patients with diabetic retinopathy in the MAVP group, 21 patients in the non-MAVP group; $P>0.05$). As shown in Table 2, sex, age, BMI, blood pressure, HbA_{1c}, serum levels of total cholesterol, folate, and vitamin B_{12} were not significantly different between diabetic patients with and without MAVP ($P>0.05$). However, the ratio of urinary Alb/Cr and serum triglycerides in patients with MAVP was significantly higher compared with the non-MAVP group ($P<0.01$ and $P<0.05$, respectively).

Plasma HcyT and Hcy Concentrations in Diabetic Patients and Controls

The distribution of plasma Hcy levels in the 40 control subjects ranged from

Table 1. Clinical characteristics and laboratory variables of diabetes patients and nondiabetic controls.

	Type 2 diabetes patients	Nondiabetic controls	*P* value
Number of patients (male/female)	120 (55/65)	40 (17/23)	0.714
Age, years, mean±SD	62.90±9.57	58.93±10.11	0.920
BMI, kg/m², mean±SD	24.95±3.24	24.26±2.79	0.444
Cholesterol, mmol/L, mean±SD	4.90±1.25	4.76±0.64	0.586
TG, mmol/L, mean±SD	2.19±1.80	1.28±0.39	<0.001
LDL, mmol/L, mean±SD	2.98±0.99	2.75±0.54	0.181
HDL, mmol/L, mean±SD	1.36±0.35	1.52±0.26	0.026
SBP, mmHg, mean±SD	137.63±21.71	123.00±9.61	<0.001
DBP, mmHg, mean±SD	78.34±12.06	81.00±6.61	0.344
FBG, mmol/L, mean±SD	8.62±3.63	4.98±0.35	<0.001
Urine Alb/Cr ratio, mg/g, median (25th and 75th quartiles)	19.50 (8-150.25)	4.10 (2.02-7.31)	<0.001
Folate, ng/mL, median (25th and 75th quartiles)	6.74 (4.78-11.25)	13.68 (12.73-15.15)	<0.001
$VitB_{12}$, pg/mL, median (25th and 75th quartiles)	480.00 (353.50-614.00)	495.00 (434.75-560.75)	0.229
Hcy, μmol/L, median (25th and 75th quartiles)	9.28 (7.51-11.82)	5.64 (5.17-8.00)	0.010
HcyT, nmol/L, median (25th and 75th quartiles)	3.38 (2.94-4.73)	2.91 (2.77-3.08)	0.023

Alb/Cr=albumin/creatinine; BMI=body mass index; DBP=diastolic blood pressure; FBG=fasting blood glucose; Hcy=homocysteine; HcyT=homocysteine thiolactone; HDL=high-density lipoprotein; LDL=low-density lipoprotein; SBP=systolic blood pressure; TG=triglycerides; $VitB_{12}$=vitamin B_{12}.

4.56 to 16.7 μmol/L. Hyperhomocysteinemia was observed in two control subjects (5%). HcyT concentrations ranged from 0 to 3.56 nmol/L, representing 0%-0.075% of plasma total Hcy in the control group. In the diabetic patients, plasma Hcy level ranged from 5.78 to 32.5 μmol/L, with 44% of patients having hyperhomocysteinemia. HcyT concentrations ranged from 0 to 25.25 nmol/L, representing 0%-0.365% of plasma total Hcy. Plasma Hcy and HcyT concentrations in type 2 diabetes patients were significantly higher than those in healthy controls (P=0.01 and P<0.05, respectively).

Plasma Hcy and HcyT levels in patients with MAVP tended to be higher compared with patients without MAVP (P<0.05 for each comparison; Table 2).

Table 2. Clinical characteristics and laboratory variables in type 2 diabetes patients with and without macrovasculopathy.

	With MAVP	Without MAVP	*P* value
Gender, male/female	28/35	27/30	0.891
Age, years, mean±SD	64.43±9.91	61.21±9.04	0.210
Smoker, no/yes	45/18	42/15	0.409
Duration of diabetes, years, median (25th and 75th quartiles)	12.00 (6.00-16.00)	11.00 (8.00-14.25)	0.745
BMI, kg/m^2, mean±SD	24.88±3.35	25.06±3.17	0.770
Ch, mmol/L, mean±SD	4.75±1.24	5.05±1.26	0.207
TG, mmol/L, mean±SD	2.61±0.36	1.80±0.98	0.021
LDL, mmol/L, mean±SD	2.83±0.95	3.12±1.02	0.122
HDL, mmol/L, mean±SD	1.34±0.39	1.38±0.30	0.642
SBP, mmHg, mean±SD	140.32±20.72	134.82±22.73	0.173
DBP, mmHg, mean±SD	77.98±13.20	78.71±10.86	0.748
Urine Alb/Cr ratio, mg/g, median (25th and 75th quartiles)	22.50 (9.00-202.00)	13.00 (8.00-110.50)	0.009
HbA_{1C}, %, mean±SD	8.42±0.87	8.08±1.79	0.324
Fibrinogen, g/L, mean±SD	3.69±1.40	3.24±0.86	0.080
Uric acid, μmol/L, mean±SD	298.48±78.66	318.65±101.35	0.235
$VitB_{12}$, pg/mL, median (25th and 75th quartiles)	488.0 (364.5-600.7)	430.0 (335.0-553.0)	0.418
Folate, ng/mL, median (25th and 75th quartiles)	8.04 (4.70-11.58)	7.67 (5.27-11.82)	0.283
HcyT, nmol/L, median (25th and 75th quartiles)	4.27 (3.02-5.11)	3.12 (2.63-3.77)	0.023
Hcy, μmol/L, median (25th and 75th quartiles)	10.36 (7.67-12.45)	7.85 (6.76-10.52)	0.026

Alb/Cr=albumin/creatinine; BMI=body mass index; Ch=cholesterol; DBP=diastolic blood pressure; FIB=fibrinogen; HbA_{1C}=glycosylated hemoglobin; HDL=high-density lipoprotein; LDL=low-density lipoprotein; Hcy=homocysteine; HcyT=homocysteine thiolactone; MAVP=macrovasculopathy; SBP=systolic blood pressure; TG=triglycerides; $VitB_{12}$=vitamin B_{12}.

Table 3. Variables associated with macrovasculopathy in type 2 diabetes patients, as measured using logistic regression analysis.

	DF	Beta	SE	Wald	*P* value	Exp (B)
Gender	1	−7.413	4.461	2.762	0.097	0.001
BMI	1	0.819	0.442	3.434	0.064	2.269
Smoking	1	8.208	4.148	3.915	0.048	3.671
Age	1	0.234	0.139	2.841	0.092	1.264
TG	1	3.020	1.504	4.033	0.045	0.049
Urine Alb/Cr ratio	1	0.173	0.087	3.928	0.047	0.841
SBP	1	0.110	0.065	2.844	0.092	1.116
HcyT	1	0.648	0.301	4.634	0.031	1.912
Hcy	1	0.791	0.380	4.340	0.037	2.206
Constant	1	−35.754	20.270	3.111	0.078	<0.001

Alb/Cr=albumin/creatinine; BMI=body mass index; DF=degrees of freedom; Exp(B)=odds ratio; Hcy=homocysteine; HcyT=homocysteine thiolactone; SE=standard error; SBP=systolic blood pressure; TG=triglycerides.

Relationship Between Plasma HcyT and Other Biochemical Parameters

Plasma HcyT concentrations were positively correlated with the ratio of urinary Alb/Cr (r=0.285, P=0.007), duration of diabetes (r=0.249, P=0.019), age (r=0.233, P=0.028), and fibrinogen (r=0.289, P=0.034), and negatively correlated with HDL levels (r=−0.223, P=0.037) in diabetic patients. However, there were no significant correlations between plasma HcyT, folate (r=−0.141, P=0.188), and vitamin B_{12} (r=−0.021, P=0.842). There was a near-significant relationship between Hcy and HcyT (r=0.211, P=0.057). Plasma Hcy levels were positively correlated with age (r=0.211, P=0.030), systolic BP (r=0.269, P<0.001), ratio of urinary Alb/Cr (r=0.327, P=0.001), uric acid (r=0.225, P=0.023), creatine (r=0.483, P<0.001), fasting blood glucose (r=0.236, P=0.017), and negatively correlated with folate (r=−0.294, P=0.003) and vitamin B_{12} (r=−0.328, P=0.001) in diabetic patients.

Risk Factors Associated with Diabetic MAVP

Stepwise binary logistic regression analysis showed that HcyT, Hcy, smoking, serum triglycerides, and ratio of urinary Alb/Cr were significantly associated with the presence of MAVP in diabetic patients (P<0.05; Table 3).

Calibration Lines for HcyT Assays

Figure 1 shows a typical standard line obtained for the analysis of HcyT over the concentration ranges from 0.1 to 1000 nmol/L. The assay is linear in the concen-

Figure 1. A typical standard calibration line obtained for the analysis of homocysteine thiolactone over the concentration range 0.1-1000 nmol/L.

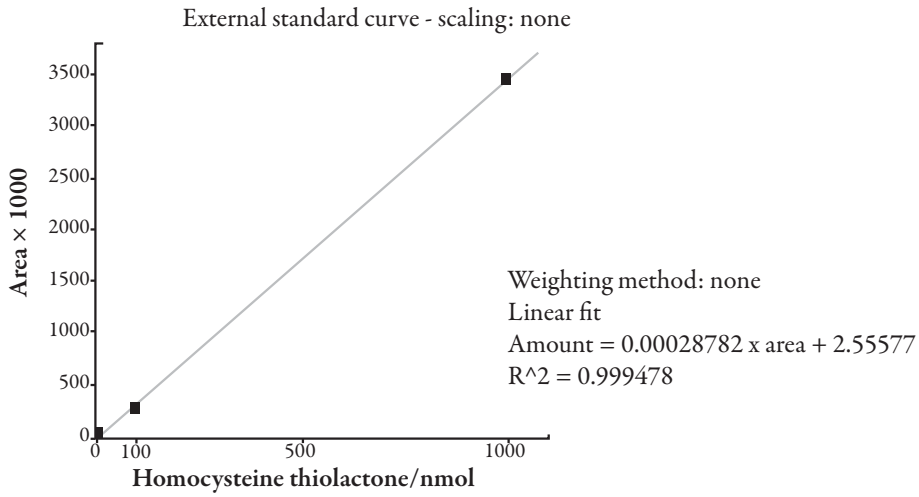
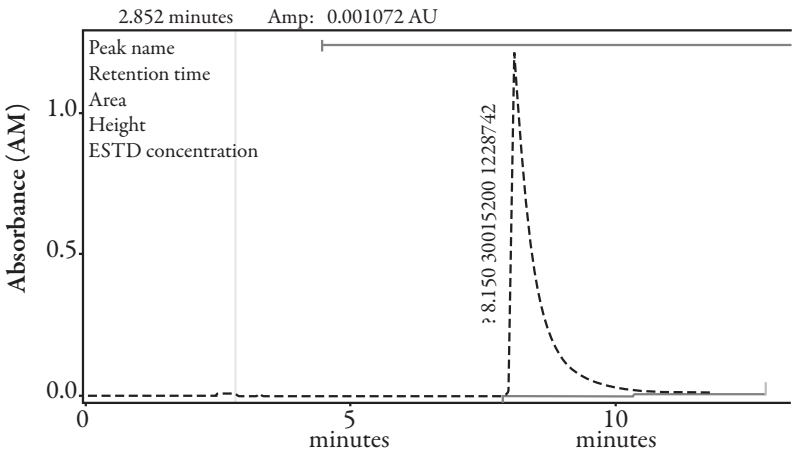


Figure 2. Reverse-phase high-pressure liquid chromatography analysis of 10 mmol/L homocysteine thiolactone standard. ESTD=external standard.

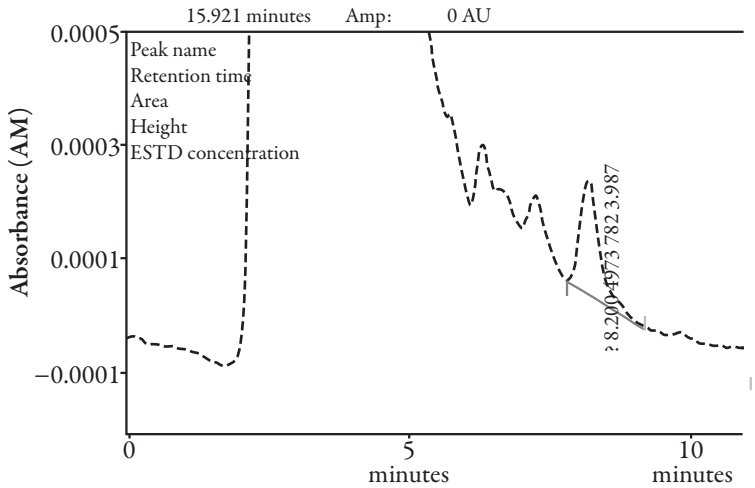


tration range. Figure 2 shows reverse-phase HPLC analysis of 10 mmol/L HcyT standard. HcyT was eluted at 8.15 minutes and there is a complete resolution of the HcyT peak. Figure 3 shows reverse-phase HPLC analysis of HcyT in human plasma using a sample from a diabetic patient. The peak of HcyT eluted at 8.15 minutes.

DISCUSSION

In this study, the major finding was that plasma HcyT and Hcy levels in diabetic patients with MAVP were significantly higher than in controls and diabetic patients without MAVP. Stepwise logistic regression analysis demonstrated that HcyT and Hcy

Figure 3. Reverse-phase high-pressure liquid chromatography analysis of homocysteine thiolactone in human plasma using a sample from a diabetic patient. ESTD=external standard.



were significantly related to MAVP, in addition to traditional risk factors in diabetic patients, indicating that HcyT and Hcy might be involved in the pathogenesis of atherosclerosis.

HcyT was serendipitously discovered in 1934.[11] The biological significance of HcyT was focused on the discovery that HcyT was a reactive product of Hcy metabolized by methionyl-tRNA synthetase (MetRS) in all cell types.[12] Jakubowski has proposed that HcyT might be the molecular basis of Hcy-induced vascular damage.[5] This hypothesis was supported by several studies.[6] It has been demonstrated that HcyT exerts its damaging effect on vascular cells in either a direct or indirect manner. In the study by Haker et al. chronic infusions of HcyT caused arterial thrombosis in baboons.[13] HcyT induced apoptotic damage in cultured human vascular endothelial cells mediated by increased intracellular hydrogen peroxide.[14] Recent studies have indicated that the toxicity of HcyT might be related to its ability to homocysteinylate proteins, which were generated by forming isopeptide bonds with side chain amino groups of protein lysine residues.[15] Alterations of structural and functional properties have been observed in Hcy-proteins.[16] Previous studies have demonstrated that plasma lipoproteins are susceptible to homocysteinylation.[16] Homocysteinylation of low-density lipoproteins (LDL) has been found to increase the atherogenicity of LDL, including facilitating the formation of small dense LDL, increasing the internalization of LDL by macrophages, and inducing the formation of thromboxane B2 and prostacyclin 6-keto-PGF during thrombosis.[17] Injection with LDL modified with HcyT induced an immune response in rabbits.[17] Xu Yang[18] has found that high levels of plasma HcyT adducts were associated with CHD. Together, these studies suggested that HcyT might represent the possible molecular mechanism underlying Hcy toxicity.

The methods for assaying HcyT in human plasma were only recently described. Some preliminary studies on HcyT in human plasma have been carried out; however, HcyT levels were highly variable among human subjects.[10,19,20] In one study with six plasma samples median HcyT was 35 nmol/L and represented 0.8% of plasma Hcy.[10] One study found that median HcyT was 21.5 nmol/L and represented 0.2% of plasma Hcy in two subjects.[19] Another study including 20 healthy individuals found the median plasma HcyT was 0.56 nmol/L and represented 0.04% of HcyT.[20] However, in our study, the median HcyT concentrations were 2.91 nmol/L, representing from 0% to 0.075% of plasma total Hcy in the 40 control subjects. The most likely reason for the apparent discrepancy between the HcyT concentrations measured in previous studies and those found in the present study might be the very limited number of samples analyzed in previous studies.

HcyT could be synthesized by MetRS in human vascular endothelial cells. Studies in vitro showed that the extent of thiolactone formation was positively correlated with concentrations of Hcy, and negatively correlated with the concentrations of methionine, folic acid, and HDL.[21] Studies have shown that HcyT hydrolase levels are associated with serum HDL.[22] Antifolates, such as aminopterin, could enhance synthesis of HcyT. In our study, results showed that plasma HcyT concentrations were positively correlated with the ratio of urinary Alb/Cr, duration of diabetes, age, and fibrinogen, and negatively correlated with HDL levels in diabetic patients. The relationship between HcyT and Hcy in human plasma has not been found in previous studies. In this study, however, there was a weak correlation between HcyT and Hcy, and the correlations between plasma HcyT and folate or vitamin B_{12} were not found, suggesting that the metabolism of HcyT is more complicated in vivo than in vitro. In addition to Hcy and HDL, there might be more factors determining plasma HcyT levels in diabetic patients, such as urinary Alb/Cr, or disease duration.

In conclusion, the present study showed that HcyT was related to diabetic MAVP in a limited number of type 2 diabetic patients. There may be more determinants responsible for HcyT concentrations in vivo than in vitro. Since HcyT has been implicated as the molecular basis of Hcy-induced vascular damage, large-scale prospective studies are needed to confirm the role of HcyT in the development of diabetic MAVP.

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