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80	Abstract	The purpose of this study was to investigate plasma homocysteine (Hcy) levels in patients with systemic sclerosis (SSc) and to study the association between plasma Hcy, C677T polymorphism of 5,10-methylenetetrahydrofolate reductase (MTHFR), and the clinical manifestations in SSc. Associations of Hcy level, C677T MTHFR polymorphism, and macrovascular diseases were investigated in 152 patients with SSc and 58 controls. No significant differences in Hcy levels and MTHFR genotypes were found in SSc patients compared to controls or in SSc patients with limited cutaneous compared to diffuse disease. Significantly higher Hcy concentration was observed in patients with macroangiopathy/thromboembolic events compared to patients without such clinical manifestations ($p < 0.05$). There was significant correlation between age and macrovascular disorders, between Hcy level and the disease duration ($r = 0.164$; $p < 0.05$). Seventy-one percent of patients with macrovascular disorders had MTHFR polymorphism. In addition, 45% of patients with hyperhomocysteinemia had pulmonary hypertension. The presence of MTHFR C677T mutation influences the incidence of macrovascular abnormalities in SSc patients. Elevated Hcy levels may be associated with disease duration and the evolution of macrovascular disorders and pulmonary hypertension in SSc.	
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Plasma Homocysteine Levels, The Prevalence of Methylenetetrahydrofolate Reductase Gene C677T Polymorphism and Macrovascular Disorders in Systemic Sclerosis: Risk Factors for Accelerated Macrovascular Damage?

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Abstract The purpose of this study was to investigate plasma homocysteine (Hcy) levels in patients with systemic sclerosis (SSc) and to study the association between plasma Hcy, C677T polymorphism of 5,10-methylenetetrahydrofolate reductase (MTHFR), and the clinical manifestations in SSc. Associations of Hcy level, C677T MTHFR polymorphism, and macrovascular diseases were investigated in 152 patients with SSc and 58 controls. No significant differences in Hcy levels and MTHFR genotypes were found in SSc patients compared to controls or in SSc patients with limited cutaneous compared to diffuse disease. Significantly higher Hcy concentration was observed in patients with macroangiopathy/thromboembolic events compared to patients without such clinical manifestations ($p<0.05$). There was significant correlation between age and macrovascular disorders, between Hcy level and the disease duration ($r=0.164$; $p<0.05$). Seventy-one percent of patients with macrovascular disorders had MTHFR polymorphism. In addition, 45% of patients with hyperhomocysteinemia had pulmonary hypertension. The presence of MTHFR C677T mutation influences the incidence of macrovascular abnormalities in SSc patients. Elevated Hcy levels may be associated with disease duration and the evolution of macrovascular disorders and pulmonary hypertension in SSc.

Keywords Systemic sclerosis · Folate · Homocysteine · Vascular disease

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Introduction

Systemic sclerosis (SSc) is associated with endothelial cell dysfunction, where classically the microvasculature is affected. Recently, it is recognized that large-vessel disease also occurs with higher incidence [1–2].

Hyperhomocysteinemia predisposes to atherosclerosis by injuring the vascular endothelium. A genetic cause of hyperhomocysteinemia is due to a mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene. A thermolabile variant of this enzyme, due to a point mutation

677°C to T changing alanine to valine, has been correlated with elevated plasma homocysteine (Hcy) levels [3].

Considering these data, we hypothesized that hyperhomocysteinemia may play a role in the pathogenesis of micro- and macrovascular damage underlying SSc. Therefore we investigated the frequency of hyperhomocysteinemia in SSc and analyzed the association of plasma Hcy levels, MTHFR C677T mutation, and clinical manifestations in our patients compared to healthy subjects.

Methods

Patients One hundred fifty-two SSc patients (131 lcSSc, 21 dcSSc) were included in our study (all satisfied the American College of Rheumatology criteria for SSc [4, 5]; mean age, 54.2 years; mean disease duration, 9.61 years; 133 females; 19 males). The classification of the patients' disease as diffuse or limited (dcSSc or lcSSc) was established according to Le Roy et al. [5]. Patients with skin sclerosis limited to hands, forearms, legs below the knee, and face were defined as having limited cutaneous scleroderma (lcSSc). Those with more extensive skin disease spreading proximal to elbows or knees or involving the trunk were classified as diffuse cutanous scleroderma (dcSSc). (The onset of disease was defined as the beginning of Raynaud's phenomenon (RP) or awareness of numbness, puffiness or sclerosis of fingers without preceding RP. Pulmonary involvement (pulmonary fibrosis or alveolitis) was defined as present by radiographic findings including X-ray and HRCT, as well as pulmonary function tests. Esophageal involvement was assessed by barium swallow test (esophageal dismotility). Cardiac involvement including relaxation and/or conduction disturbances, right ventricular hypertrophy were assessed by 2-D as well as Doppler echocardiography and ECG. For comparison, we studied 58 age- and sex-matched healthy controls (mean age, 49.9 years; 46 females and 12 males).

Laboratory tests Total Hcy levels were measured with high-performance liquid chromatography method (BIO-RAD, Hercules, CA, USA) [6]. The upper limit of the normal range of total plasma homocysteine was 12.5 μmol/l.

The C677T mutation of the MTHFR gene was assessed by DNA-fragmentation using specific restriction endonuclease enzyme followed by PCR amplification and agarose gel electrophoresis [7]. Patients and controls were genotyped as homozygous for the mutation (TT), heterozygous (CT), or wild-type (CC).

Statistical analysis Statistical analysis included paired *t* test, Mann–Whitney *U* test, calculation of Spearman's correlation coefficient, and Chi-square test was applied to compare parameters in different groups. p values<0.05 were considered significant.

Results

Seventy-eight percent of SSc patients had pulmonary, 57% had esophageal and 39% had myocardial involvement, while 9.8% of the patients had pulmonary hypertension (PAH) as screened by Doppler echocardiography. PAH was considered present if the estimated right ventricular systolic pressure exceeded 45 mmHg. Twenty percent of SSc patients had macrovascular manifestations (24 lcSSc, seven dcSSc). Twenty-six of 31 patients (84%) had obliterative arteriosclerosis of lower extremities, eight (26%) had coronary heart disease, two (6.4%) had stroke, and three (9.7%) had deep venous thrombosis. Macrovascular diseases of SSc patients and cardiovascular risk factors (hypertension, diabetes mellitus, hyperlipoproteinemia, and smoking) showed no strong correlation except between hypertension and cerebral stroke. Main cardiovascular risk factors of patients and controls are shown in Table 1.

The mean plasma Hcy levels were 9.3 μmol/l in SSc and 10.1 μmol/l in controls. There were no significant differences in the Hcy levels between SSc and controls or between lcSSc or dcSSc subtypes.

Analyzing the MTHFR genotypes, no statistical differences were found between SSc patients and controls. Forty-nine percent of patients showed wild, 36% heterozygous, and 15% homozygous MTHFR genotypes while 40% of controls had wild, 47% heterozygous, and 13% homozygous genotypes. There were no significant differences in Hcy levels between homozygous, heterozygous, and wild genotype within the Ssc and control group.

Analyzing correlations between Hcy levels and macroangiopathic/thromboembolic events in SSc we found significantly higher Hcy concentrations in patients with macroangiopathic/thromboembolic events (10.5±7.1 μmol/l) compared to patients without such clinical manifestations (9.1±7.6 μmol/l) (Fig. 1). Six of 31 patients with macroangiopathy/thromboembolic events (19%) had homozygous (TT)

Table 1 Main cardiovascular risk factors of SSc patients and controls

Risk factors	Systemic sclerosis (152)	Control (58)
BMI (kg/m^2)	22.8±2.6	24.7±4.8
Hypertension	15 (9.8%)	–
Diabetes mellitus	6 (3.9%)	–
Hyperlipoproteinemia	20 (13%)	6 (10.3%)
Smoking	1 (0.6%)	8 (13.7%)
Family history of cardiovascular disease	34 (22.37%)	20 (34.48%)

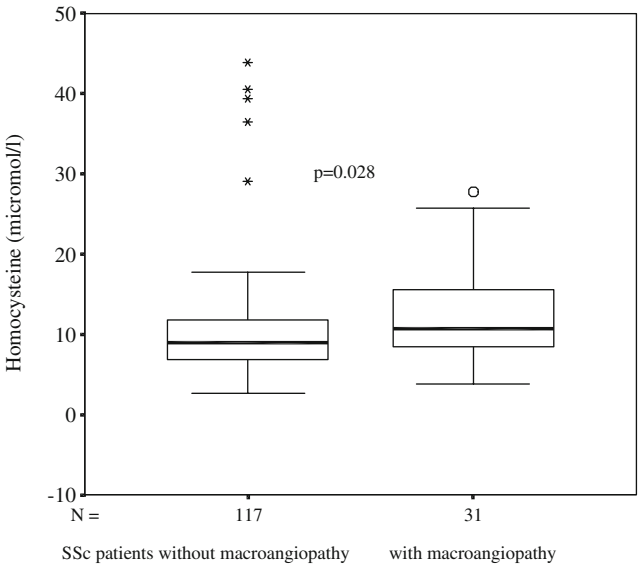

Fig. 1 Plasma homocysteine levels according to the presence of macroangiopathy in SSc patients

MTHFR variants, 16 (52%) had heterozygous (CT), and nine (29%) had wild type (CC).

A significant correlation was found between the age of SSc patients and the existence of macrovascular disorders (Fig. 2). Finally, a positive correlation was observed between plasma Hcy levels and the disease duration of SSc (r=0.164, p=0.043). In addition, the presence of macrovascular abnormalities in SSc patients was associated with longer disease duration (Fig. 3).

Analyzing the clinical parameters, prevalence of pulmonary hypertension was elevated in our patients who had higher than 15 μmol/l plasma Hcy concentration. Ten of 22 patients with

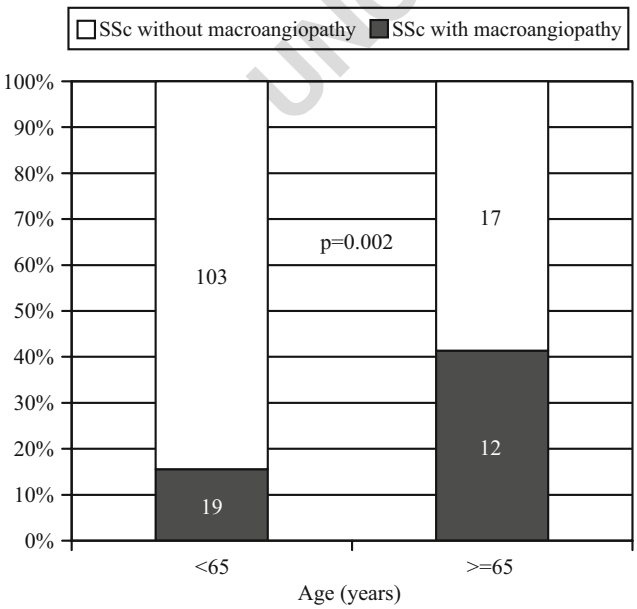

Fig. 2 Relations of age with macroangiopathy in SSc patients

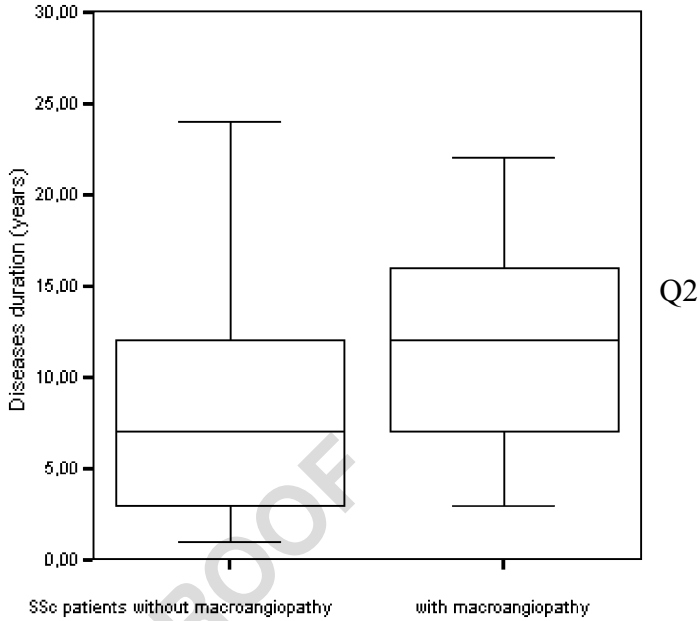

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Fig. 3 Relation of disease duration with macroangiopathy in SSc patients

Hcy concentration >15 μmol/l had pulmonary hypertension (45%). In total SSc patients there were only 15 patients (9.8%) with pulmonary hypertension. There was no correlation between Hcy concentration and other clinical or serological parameters.

Discussion

SSc is a vascular disease, where the microvasculature is affected. In recent years increased attention has been paid to the importance of large vessel involvement in SSc [2]. Increased plasma Hcy concentration is an independent risk factor for macrovascular disorders and it may be associated with an increased risk of small-vessel thrombosis also [7]. In the present study, we did not find significant differences in Hcy levels and MTHFR polymorphism between SSc patients as well as healthy controls lacking any vascular disease, although impaired endothelial function in hyperhomocysteinemia is well known [8]. The frequency of C677T variant of MTHFR gene in our results are similar to other reports where the homozygous form was found in about 10–13% and the heterozygous form in about 45% of Caucasian people [9].

Assessing the relationship between Hcy levels and the occurrence of macroangiopathic/thromboembolic events in SSc patients, we found significantly higher Hcy concentrations in patients with vascular/thromboembolic events in comparison to SSc patients without such manifestations. Altogether, 71% of patients with macrovascular disorders had either homozygous (TT) or heterozygous (CT) MTHFR

variants. These data suggest that the existence of MTHFR C677T mutation (TT or CT form), may influence the incidence of macrovascular abnormalities in SSc. Although it is the microvasculature that is primarily affected in patients with SSc, it is recognized that large-vessel disease also occurs with higher incidence and the involvement of the macrovasculature may be involved in the outcome of SSc [2]. Considering these results and data previously reported by others, which could not identify MTHFR gene polymorphism as an independent risk factor for vascular complications in macrovascular diseases, we can conclude that there are other risk factors that may be crucial for the development of macrovascular manifestations in SSc [10–11].

We found a positive correlation between age and the existence of macrovascular manifestations in SSc. Besides there was a significant correlation between disease duration and the development of macrovascular manifestations. These results suggest that macrovascular disease is not only an age-related feature in SSc but may also depend on disease-associated mechanisms. Finally, prevalence of pulmonary hypertension was elevated in 45% of our patients who had >15 μmol/l plasma Hcy concentration, which may have relevance for clinical practice.

In summary, our results suggest that hyperhomocysteinemia and the polymorphism of MTHFR gene may be involved in the vascular damage associated with SSc and further prospective studies are needed to clarify their role in endothelial dysfunction. We acknowledge that the data require further effort and recapitulation in a larger series, but we also note the critical contribution of genetics in other autoimmune situations [12–26].

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. The phrase “To investigate plasma homocysteine” was changed to “The purpose of this study was to investigate plasma homocysteine”; and the phrase “while 9.8% of the patients had pulmonary hypertension (Pulmonary hypertension (PAH) was screened by Doppler echocardiography.” was changed to “while 9.8% of the patients had pulmonary hypertension (PAH) as screened by Doppler echocardiography.” Please check if appropriate.

Q2. Figure 3 – Supplied efile contains pixilated text. Please provide better quality of figure.