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RESEARCH ARTICLE

# Plasma total homocysteine level and methylenetetrahydrofolate reductase 677C>T genetic polymorphism in Japanese patients with rheumatoid arthritis

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**Abstract**

Hyperhomocysteinemia is a known risk factor of cardiovascular disease. Homocysteine has been also linked to inflammation in rheumatoid arthritis (RA). In this study, we investigated the relationship between plasma homocysteine levels and single nucleotide polymorphism (SNP) of the gene coding for methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in the biosynthesis of homocysteine, and the correlation between the plasma homocysteine levels and generally used inflammatory markers (C-reactive protein, erythrocyte sedimentation rate and matrix metalloproteinase-3) in 96 Japanese patients with RA. Plasma homocysteine levels in patients with the *MTHFR* 677TT genotype were significantly higher than in those with the 677CC genotype ($p < 0.05$). In addition, plasma homocysteine levels were increased along with the elevation of general inflammatory markers. Therefore, we conclude that homocysteine might affect the inflammatory status of patients, and the *MTHFR* 677C>T SNP could be a predictive factor of hyperhomocysteinemia in patients with RA.

**Keywords:** *Homocysteine; inflammation; methylenetetrahydrofolate reductase; rheumatoid arthritis; single nucleotide polymorphism*

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease leading to joint inflammation that affects 0.5–1.0% of the worldwide population (Ranganathan & McLeod 2006). C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are currently used for monitoring the inflammatory status of RA. CRP and ESR are not specific markers for inflammation in RA because they have been used in many inflammatory diseases. Thus, a new marker that reflects more precisely the inflammatory status in patients with RA is needed.

Homocysteine has been linked to inflammation in RA. It was reported that the elevation of homocysteine levels correlates with the markers of inflammation in patients with RA (Chiang et al. 2003, Yxfeldt et al. 2003). Homocysteine is an amino acid containing the thiol group and is an intermediate product of the metabolism of methionine. It has been known that homocysteine levels are influenced by nutrients such as folic acid and the vitamin B complex, and anti-RA drugs such

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as methotrexate (MTX) and salazosulfapyridine (SSZ) (Haagsma et al. 1999, Chiang et al. 2003, Dierkes & Westphal 2005). Recent studies have reported on homocysteine in metabolic syndrome (Bellia et al. 2007), and some studies have reported a correlation between plasma homocysteine levels and the risk of cardiovascular disease (Brattstrom et al. 1998, Eikelboom et al. 1999, Klerk et al. 2002, Lewis et al. 2005).

Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) is an enzyme involved in the biosynthesis of homocysteine. MTHFR is expressed in organs such as the brain, small intestine, liver and kidney (Human Protein Reference Database, http://www.hprd.org/index_html) and is one of the major enzymes in the folate metabolic pathway (Krajinovic & Moghrabi 2004). In the process of folate metabolism, homocysteine is converted into methionine by receiving the methyl group from 5-methyltetrahydrofolate (5-$CH_3$-THF). MTHFR catalyzes the reaction of 5,10-methylenetetrahydrofolate (5,10-$CH_2$=THF) to 5-$CH_3$-THF (Kang et al. 1993, Ueland et al. 2001, Ranganathan et al. 2003, Krajinovic & Moghrabi 2004, Ranganathan & McLeod 2006). Thus, if the level of 5-$CH_3$-THF were low, homocysteine would accumulate in the cells (Krajinovic & Moghrabi 2004, Martin et al. 2006).

Two single nucleotide polymorphisms (SNPs) of *MTHFR* 677C>T and 1298A>C have been widely studied among the many polymorphisms in the *MTHFR* gene. In particular, it is reported that *MTHFR* 677C>T, which substitutes alanine for valine, affects plasma homocysteine levels (Ueland et al. 2001, Ranganathan et al. 2003, Ranganathan & McLeod 2006). The thermolability of MTHFR resulting from *MTHFR* 677C>T SNP causes decreased enzyme activity, followed by an increase in plasma homocysteine levels (Kang et al. 1993, Martin et al. 2006, Ranganathan & McLeod 2006). In the Japanese population, the distribution of *MTHFR* 677C>T SNP is estimated as follows: 30–40% are wild type (677CC), approximately 50% are heterozygous type (677CT) and 10–20% are mutant type (677TT) (Inoue et al. 2007). Although the relationship between plasma homocysteine levels and the risk of cardiovascular diseases, and the adverse reaction to anti-RA drugs have been explored by several researchers (Eikelboom et al. 1999, Kang et al. 1993), the relationship between the inflammation status, plasma homocysteine levels and the genetic polymorphisms in folate-metabolizing enzymes in patients with RA is still unclear.

In this study, we investigated the relationship between plasma homocysteine levels and *MTHFR* 677C>T SNP, and the relationship between plasma homocysteine levels and the inflammatory status in Japanese patients with RA. We found elevated plasma homocysteine levels in *MTHFR* 677TT patients and that plasma homocysteine levels may affect the inflammatory status of Japanese patients with RA.

## Materials and methods

### *Study population*

Ninety-six Japanese patients with RA were enrolled in this study. RA was diagnosed according to American College of Rheumatology criteria. We obtained informed consent from all the patients, and this study was approved by the ethics committee of the Shizuoka Kousei Hospital.

### *Sample collection*

Whole venous blood of subjects was collected using vacuum collection tubes. Plasma was obtained by immediate centrifugation from a part of collected whole venous blood and stored at –80°C until analysis. The venous blood was aliquoted in Eppendorf tubes and stored at –80°C until analysis.

### *Measurement of plasma homocysteine levels*

Plasma homocysteine was measured by enzymatic assay using the Enzymatic Homocysteine Assay (AXIS-SHIELD, Dundee, UK) according to the manufacturer's instructions (manual measurement). The calibration was performed using the Enzymatic Homocysteine Control Kit (AXIS-SHIELD) and the 96-well microplate reader (Wallac 1420 ARVOsx; Perkin Elmer, Waltham, MA, USA) for measurement of absorbance at 340 nm before measurement of each sample.

### *Genotyping of MTHFR 677C>T*

Genomic DNA was extracted from whole blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). *MTHFR* 677C>T polymorphism (rs1801133) was determined by polymerase chain reaction (PCR) – restriction fragment length polymorphism (RFLP) as reported earlier (Yi et al. 2002) with minor modifications. The PCR solution (final volume of 20 µl) contained 30–50 ng of genomic DNA at a final concentration of 0.5 µmol $l^{-1}$ each of forward primer (5′-TGA ACA GGT GGA GGC CAG CCT CT-3′) and reverse primer (5′-AGG ACG GTG CGG TGA GAG TG-3′), a 50 µmol $l^{-1}$ dNTP mixture, 1.5 mmol $l^{-1}$ $MgCl_2$ and 0.5 units HotStarTaq DNA polymerase (Qiagen). PCR amplification was performed in a thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA) for initial denaturation at 95°C for 15 min, 35 cycles (95°C, 15 s; 65°C, 30 s; and 72°C, 45 s)

and a final extension at 72°C for 10 min. The amplified product (299 bp) was digested with 2.5 U of *Hinf*I (New England BioLabs, Ipswich, MA, USA) at 37°C. Digested products were analyzed on 3% agarose gels.

### *Inflammatory markers*

To evaluate the inflammatory status in patients with RA, we obtained the clinical values of CRP, ESR and matrix metalloproteinase 3 (MMP-3) from the medical records of each patient.

### *Statistics*

Significant differences in plasma homocysteine levels between and the *MTHFR* 677CC and CT genotypes and between CC and TT genotypes were analyzed by an unpaired *t*-test (significance level $p = 0.05$, two-sided). The correlation between plasma homocysteine levels and the clinical values of inflammatory markers were tested by regression analysis using Pearson's correlation coefficient (GraphPad Prism 4 software).

## Results

### *Patient characteristics*

Patient characteristics are shown in Table 1. The distribution of *MTHFR* 677C>T genotypes was as follows: 39 patients (40.6%) had 677CC (wild type), 42 (43.8%) had 677CT (heterozygote) and 15 (15.6%) had 677TT (mutant type). The genotype distribution agreed with that expected by the Hardy–Weinberg equilibrium. The statistical difference between the genotype distributions in this study and those in a previously reported study of the Japanese population (Inoue et al. 2007) was not significant. In addition, plasma homocysteine levels were not affected by treatment and dose of anti-RA drugs and folic acid supplementation.

### *Association between plasma homocysteine levels and MTHFR 677C>T*

Plasma homocysteine levels in patients with RA were compared with their *MTHFR* 677C>T genotype (Figure 1). Plasma homocysteine levels in patients with the *MTHFR* 677TT genotype were significantly higher than in those with the 677CC genotype (*MTHFR* 677CC vs 677TT genotype, unpaired *t*-test; $p < 0.05$). Plasma homocysteine levels in patients with the *MTHFR* 677CT genotype were not significantly different from those in those with the 677CC and 677TT genotypes.

**Table 1.** Patient's characteristics.

| | 677CC ($n = 39$) | 677CT ($n = 42$) | 677TT ($n = 15$) |
|---|---|---|---|
| Age (years), median (range) | 63 (40–87) | 61 (35–89) | 64 (46–80) |
| Sex: male/female | 6/33 | 7/35 | 2/13 |
| MTX administration, *n* | 30 | 32 | 13 |
| Dose (mg weekly), median (range) | 6 (2–10) | 6 (2–8) | 6 (4–8) |
| Folic acid administration, *n* | 15 | 11 | 2 |
| Dose (mg weekly), median (range) | 10 (5–10) | 10 (10–20) | 10 (10–20) |
| SSZ administration, *n* | 10 | 6 | 6 |
| Dose (mg daily), median (range) | 1000 (500–1000) | 1000 (500–1000) | 1000 (500–1000) |
| Oral corticosteroid, *n* | 27 | 23 | 13 |
| Prednisolone dose[a] (mg daily), median (range) | 5 (1–10) | 3 (1–8) | 5 (2–8) |
| NSAIDs treatment, *n* | 31 | 30 | 10 |
| Anti-TNF-α therapy, *n* | 3 | 4 | 3 |
| Infliximab | 1 | 1 | 1 |
| Etanercept | 2 | 3 | 2 |
| Other DMARDs treatment (*n*) | 7 | 15 | 2 |
| Sodium aurothimalate | 5 | 8 | 0 |
| Auranofin | 1 | 3 | 0 |
| Actarit | 0 | 2 | 1 |
| Bucillamine | 1 | 0 | 1 |
| Penicillamine | 0 | 2 | 0 |
| Other drugs for RA treatment, *n* | 3 | 1 | 1 |
| Azathioprine | 1 | 0 | 0 |
| Tacrolimus hydrate | 2 | 1 | 1 |

[a]The dose of oral corticosteroid was converted into its of prednisolone.

There was no significant difference among the genotypes in all the data above.

MTX, methotrexate; SSZ, salazosulfapyridine; NSAIDs, non-steroidal anti-inflammatory drugs; TNF, tumour necrosis factor; DMARDs, disease-modifying antirheumatic drugs; RA, rheumatoid arthritis.

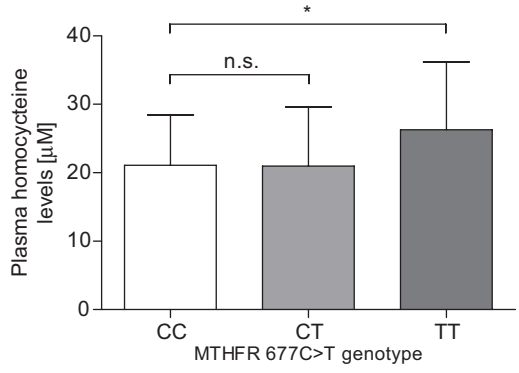


**Figure 1.** Plasma homocysteine levels in each genotype of *MTHFR* 677C>T. Plasma homocysteine levels (μM) are shown as mean ± SD. The number of patients is as follows: 39 CC, 42 CT and 15 TT. *Significant difference by unpaired *t*-test ($p < 0.05$); n.s., not significant.

### *Correlation between general inflammatory markers and plasma homocysteine levels*

The relationships between plasma homocysteine levels and general inflammatory markers (CRP, ESR and MMP-3) are shown in Figures 2, 3 and 4. Plasma homocysteine levels were significantly increased along with the elevation of these inflammatory markers. Plasma homocysteine levels may reflect the severity of inflammation in patients with RA.

## Discussion

In this study, we investigated the correlation between plasma homocysteine levels and *MTHFR* 677C>T SNP, and the relationship between plasma homocysteine levels and the inflammatory status in Japanese patients with RA. It has been thought that elevated levels of plasma homocysteine resulted from *MTHFR* 677C>T SNP. Previous studies (Haagsma et al. 1999, Ueland et al. 2001, Krajinovic & Moghrabi 2004) as well as this study support this hypothesis. It has been suggested that *MTHFR* 677C>T SNP is one factor that influences plasma homocysteine levels, in addition to others such as age and dietary intake (Ueland et al. 2001). A number of groups reported that elevated levels of plasma homocysteine were detected only in the case of *MTHFR* 677T homozygotes (Brattstrom et al. 1998, Ueland et al. 2001). These observations suggest that only homozygotes of *MTHFR* 677T may pose a risk for hyperhomocysteinemia and that heterozygotes are not an important risk for hyperhomocysteinemia and may be a recessive inheritance following *MTHFR* 677C>T SNP.

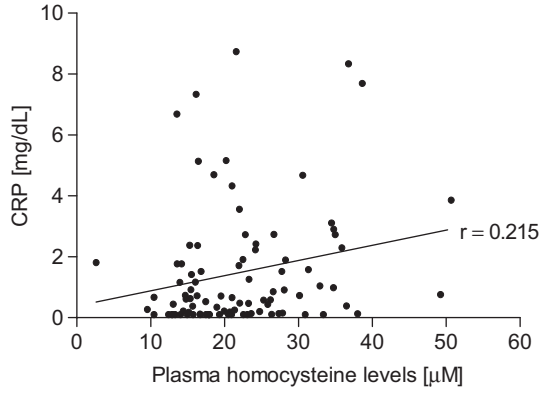


**Figure 2.** Correlation between C-reactive protein (CRP) (mg $dl^{-1}$) and plasma homocysteine (μM) levels. The levels of CRP obtained from 92 patients are shown. Pearson's correlation coefficient is 0.215 (95% confidence interval 0.0111–0.403; $P = 0.039$).

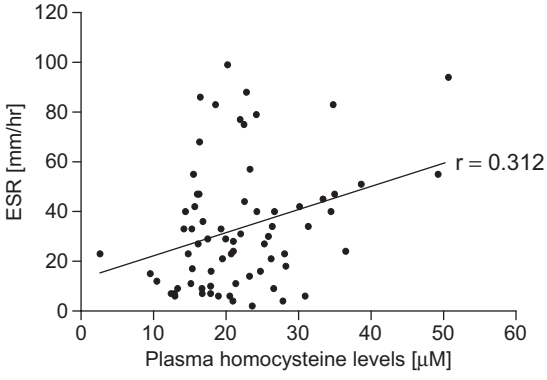


**Figure 3.** Correlation between erythrocyte sedimentation rate (ESR) (mm $h^{-1}$) and plasma homocysteine (μM) levels. ESR levels obtained from 70 patients are shown. Pearson's correlation coefficient is 0.312 (95% confidence interval 0.0827–0.509; $P = 0.009$).

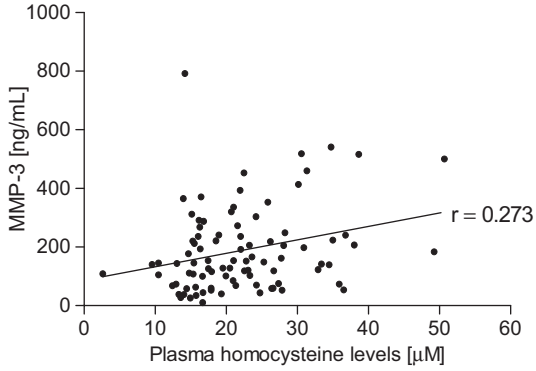


**Figure 4.** Correlation between matrix metalloproteinase (MMP)-3 (ng $ml^{-1}$) and plasma homocysteine (μM) levels. MMP-3 levels obtained from 89 patients are shown. Pearson's correlation coefficient is 0.273 (95% confidence level 0.0686–0.455; $P = 0.01$)

It was reported that in patients with RA, plasma homocysteine levels were elevated by MTX administration, although this elevation was partly counteracted by administration of folic acid in 113 patients with RA by a randomized control study (van Ede et al. 2002). It was reported that the circulating levels of vitamin B complex, included folic acid, were low in patients with RA, and the homocysteine level was high in patients with RA (Roubenoff et al. 1997, Woolf & Manore 2008). However, in our study, there was no significant difference in the homocysteine levels between the subjects administered MTX and those not receiving it ($p = 0.181$), and between the subjects given a folate supplement and those not receiving the supplement ($p = 0.194$) (data not shown). Therefore, we analyzed the data in terms of the inflammation and the homocysteine levels without classifying the patients with RA according to the intake of MTX and folic acid, because the inflammatory status might be the important factor that affects plasma homocysteine levels.

Earlier researchers have reported the relationship between homocysteine levels and the inflammation status in patients with RA. It has been reported that homocysteine increased the concentration of the

inflammatory cytokines interleukin (IL)-6 and IL-8, and contributes to the activation of NF-κB, one of the inflammatory transcription factors, through experiments using cultured synoviocytes derived from patients with RA, but not from healthy subjects (Lazzerini et al. 2006). We consider that in healthy subjects, homocysteine is not a risk factor for inflammation. Chiang et al. (2003) reported a correlation between plasma homocysteine levels and CRP and/or ESR in patients with RA. Our results agree with those results. Moreover, we found that the plasma homocysteine levels were increased along with the elevation of MMP-3 levels. MMP-3 is an indicator of synoviocyte proliferation. It is also known to be elevated in autoimmune/inflammatory diseases such as systemic lupus erythematosus (Momohara & Yamanaka 2005, Tso et al. 2006). MMP-3 is considered to be a useful marker for predicting the outcome of joint destruction in RA and reflects RA-caused inflammation status more specifically than CRP and ESR. Their observations indicate that MMP-3 is a more suitable marker for predicting the disease course of RA than CRP or ESR. The correlation of MMP-3 and plasma homocysteine (Figure 4) indicates that homocysteine may be applicable as a marker for activity of RA. Recently, the 'homocysteine inflammatory loop' was proposed by Lazzerini et al. (2007). They suggested that homocysteine activates NF-κB to enhance cytokine production, and that immunoinflammatory activation might enhance turnover of immune cells leading to vitamin depletion and hyperhomocysteinemia. According to their hypothesis, hyperhomocysteinemia might not only be the result of inflammation, but also cause the elevation of the inflammatory level in RA. From this point of view, it is anticipated that homocysteine would increase initially in the inflammatory process, and maintain high levels in the inflammatory state. In our study, there is no direct association between the *MTHFR* 677C>T genotype and inflammation levels. However, we consider that the *MTHFR* 677C>T genotype is indirectly associated with the inflammatory levels because the *MTHFR* 677C>T genotype affects plasma homocysteine levels and plasma homocysteine levels related to the inflammatory levels.

The relationship between homocysteine and cardiovascular disease has been reported by some groups (Brattstrom et al. 1998, Whittle and Hughes 2004). Plasma homocysteine levels depend on *MTHFR* 677C>T SNP in patients with certain cardiovascular diseases (Eikelboom et al. 1999, Bellia et al. 2007). *MTHFR* 677C>T SNP also causes elevation of plasma homocysteine levels in patients with RA (Figure 1). The association between *MTHFR* 677C>T SNP and adverse reactions to anti-RA therapy has been studied because many patients with RA experience complications associated with cardiovascular disease (Haagsma et al. 1999, van Ede et al. 2001, Urano et al. 2002, Kumagai et al. 2003, Berkun et al. 2004). From this point of view, homocysteine might not only reflect the inflammatory status in patients with RA but also predict the efficacy and adverse reactions of anti-RA therapy. Homocysteine might be a key biomarker in the follow-up of RA.

We consider that homocysteine may affect the elevation of the inflammatory level of RA. More detailed studies are needed to elucidate how homocysteine affects the inflammation status of patients with RA. A prospective cohort study is necessary to confirm this assumption.

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