Decreased expression of the *methylene tetrahydrofolate reductase* (*MTHFR*) gene in patients with rheumatoid arthritis

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Abstract

Objective

Impairment of methylene tetrahydrofolate reductase (MTHFR), a key enzyme in the folate metabolism, results in an elevated plasma level of homocysteine, considered an independent risk factor for cardiovascular (CV) disease. Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with increased risk of CV death. Polymorphisms in the MTHFR gene increase the frequency of CV disease in RA. The aim of this study was to determine the expression of MTHFR gene in patients with RA, with and without ischaemic heart disease (IHD).

Methods

Relative expression of MTHFR gene and beta-actin and GAPDH as housekeeping genes was quantified by quantitative real-time polymerase chain reaction. It was analysed by the comparative Ct (threshold cycle) method in peripheral blood from 26 Spanish patients with RA (12 with IHD and 14 without IHD) and 10 healthy controls. MTHFR expression level in RA patients was also assessed according to disease activity, rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies status.

Results

MTHFR expression was significantly reduced in patients with RA compared to controls (fold change = 0.85, p=0.029). It was especially true for RA patients with IHD (fold change= 0.79, p=0.021). However, no statistically significant relationship between MTHFR expression level in patients with RA and DAS28 CRP, DAS28 ESR, RF and anti-CCP status was observed.

Conclusion

Patients with RA, in particular those with IHD, show a decreased expression of the MTHFR gene. This may support a potential implication of the transcriptional regulation of MTHFR in the pathogenesis of RA.

Key words

methylene tetrahydrofolate reductase (MTHFR), rheumatoid arthritis, cardiovascular disease, ischaemic heart disease, gene expression

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Received on June 1, 2015; accepted in revised form on September 4, 2015.

Funding: this study was supported by European Union FEDER funds and Fondo de Investigación Sanitaria (grant PI12/00060) (Spain).
This work was also partially supported by RETICS Programmes RD12/0009/0013 and RD12/0009/0004 (RIER) from the Instituto de Salud Carlos III (ISCIII) (Spain), and in part by grants from the European IMI BTCure Programme.
F. Genre and B. Ubilla are supported by funds from the RETICS Programme (RIER) (RD12/0009/0013).
R. López-Mejías is a recipient of a Sara Borrell postdoctoral fellowship from the ISCIII (CD12/00425)

Competing interests: none declared.

Introduction

Methylene tetrahydrofolate reductase (*MTHFR)* is a key enzyme in the folate metabolism which catalyses the irreversible reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate, the methyl donor for the conversion of homocysteine to methionine (1). The human *MTHFR* gene is located on chromosome 1p36.3 (2). Mutant alleles of C677T and A1298C polymorphisms in the *MTHFR* gene are associated with a reduction of MTHFR enzyme activity leading to a higher risk of cardiovascular (CV) disease (3, 4). The impairment of the MTHFR enzyme to produce folate results in increased levels of homocysteine in blood. Hyperhomocysteinaemia is an independent risk factor for CV disease, including ischaemic heart disease (IHD) (5, 6) and it is present in CV and systemic inflammatory autoimmune diseases (7, 8).

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis associated with an increased risk of CV death (9), mainly due to a process of endothelial dysfunction and accelerated atherosclerosis (10, 11). IHD is the leading cause of the increased mortality in patients with RA (12, 13).

The aetiology of CV disease in RA is complex. Besides the influence of chronic inflammation and traditional and non-traditional risk factors (14-17), a genetic component also accounts for the increased risk of CV morbidity in patients with RA (18, 19). Therefore, the search for candidate genes and biomarkers of CV disease is of potential relevance to identify RA patients at risk of CV death (20, 21).

Gene expression of peripheral blood represents an approach to assess cellular changes due to disease pathogenesis. Several studies have been conducted to identify novel targets or predict patient's response to a given treatment in RA (22-26).

Methotrexate (MTX) is the mainstay of treatment in RA. Although significant survival benefit, mainly reducing CV mortality, was found in patients with RA undergoing MTX therapy (27), this medication reduces plasma and red blood cell folate levels, which increases homocysteine levels via reduced activity of the MTHFR enzyme (28, 29). Because of that, a common practice in patients with RA includes supplementation with folic acid for long-term MTX therapy, since it decreases plasma homocysteine levels preventing MTX toxicity and hyperhomocysteinaemia (30, 31).

We previously reported an association of the functional A1298C polymorphism in the *MTHFR* gene with CV events in patients with RA after 5 years and 10 years of follow-up. Moreover, patients with RA carrying the *MTHFR* 1298 AC and CC genotypes had a severe endothelial dysfunction when compared with those carrying the *MTHFR* 1298 AA genotype (32).

Taken together all these considerations, in the present study we aimed to determine the relative *MTHFR* messenger ribonucleic acid (mRNA) expression in patients with RA, with and without IHD, compared to controls.

Patients and methods

Patients and controls

Patients with RA who met the 1987 American College of Rheumatology (ACR) and the 2010 ACR/European League Against Rheumatism criteria for RA (33, 34) were recruited from Hospital Universitario Marqués de Valdecilla (Santander, Spain). We included 26 RA patients with (n=12) or without (n=14) IHD that were frequency-matched for age and duration of the disease.

Based on previously established protocols of management, all patients with MTX therapy received folic acid supplementation (5–10 mg/week).

As a control group, we included ten volunteers with no history of autoimmune or CV disease. Controls were frequency-matched for sex and age to patients.

Informed consent was obtained from all the subjects. The Ethics Committee of Clinical Research of Cantabria (Spain) approved the study. Information on the main demographic and clinical characteristics of patients and controls is shown in Table I.

Blood RNA extraction

Peripheral blood samples were obtained from the subjects and collected into Venous Blood Vacuum Collection

Table I. Demographic and clinical characteristics of healthy controls and patients with RA included in the study.

Characteristic	Controls, n=10 % (n/N)	RA patients, n=26 % (n/N)
Women	60 (6/10)	50.0 (13/26)
Age at time of study (years, mean ± SD)	61.9 ± 7.6	62.0 ± 7.0
Follow-up (years, mean ± SD)	–	8.5 ± 4.6
Rheumatoid factor positive	–	53.8 (14/26)
Anti-CCP antibodies positive	–	46.2 (12/26)
DAS28 CRP (mean ± SD)	–	2.7 ± 1.0
DAS28 ESR (mean ± SD)	–	3.0 ± 1.3
ESR (mm/hour, mean ± SD)	–	20.3 ± 18.8
Ischaemic heart disease	–	46.2 (12/26)
Traditional cardiovascular risk factors		
Hypertension	–	46.2 (12/26)
Dyslipidaemia	–	61.5 (16/26)
Current smoking	–	23.1 (6/26)
Diabetes	–	11.5 (3/26)
Obesity	10.0 (1/10)	34.6 (9/26)
Synthetic DMARD*		
Any	–	100.0 (26/26)
Methotrexate	–	88.5 (23/26)
Chloroquine/Hydroxychloroquine	–	80.8 (21/26)
Leflunomide	–	34.6 (9/26)
Biologic DMARD*		
Any	–	38.5 (10/26)
TNF-α inhibitor	–	23.1 (6/26)
Non TNF-α inhibitor	–	23.1 (6/26)
Corticosteroids*	–	96.2 (25/26)
Statins at the time of the study	–	53.8 (14/26)

SD: standard deviation; Anti-CCP: anti-cyclic citrullinated peptide antibodies; DAS: disease activity score; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DMARD: disease-modifying anti-rheumatic drug. *At the time of the study or before.

Tubes containing ethylenediaminetetraacetic acid (EDTA). Total RNA was isolated few hours after the extraction according to the manufacturer's protocol using NucleoSpin RNA Blood Midi Kit (Macherey-Nagel). Purity and concentration of total RNA was measured by ND-1000 Nanodrop Spectrophotometer (Nanodrop, Wilmington, DE, USA). Samples were concentrated using GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific) and stored at -80ºC until further processing.

Quantitative real-Time PCR

For each sample, 1 μg of total RNA was reverse transcribed into cDNA using iScriptTM Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA, USA). 20 ng of cDNA was used for quantitative real-time PCR (qPCR), using SsoAdvancedTM Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) in custom 96-well-plates (PrimePCR Assays, Bio-Rad, Hercules, CA, USA). *MTHFR* target gene and *beta-actin* and *GAPDH* as housekeeping genes were analysed. All samples were assayed in triplicate and controls (positive PCR control assay, reverse transcription control assay, DNA contamination control assay and RNA quality assay) were included. Quantitative PCR reactions were performed in a CFX-96 real-time instrument (Bio-Rad, Hercules, CA, USA) using the following conditions: stage 1: 95ºC for 2 min, stage 2: 95ºC for 5 sec followed by 60ºC for 30 sec repeated for 40 cycles. A melt curve step was added for 65-95ºC (0.5ºC increments per second). The threshold cycle (Ct) was automatically established and recorded by the CFX manager software (Bio-Rad, Hercules, CA, USA). Ct value represents the cycle number in which the PCR products reach the threshold level. The relative *MTHFR* expression was analysed by the comparative Ct method. First, delta Ct (ΔCt) was calculated by subtracting the average Ct values for *MTHFR* from the average Ct values of the housekeeping genes. Next, the double delta Ct (ΔΔCt) for each sample was calculated as 2-ΔΔCt. The normalised expression level was deducted for each sample and these values were used to calculate averages for each study group. The fold-change (changed amount of expression) of *MTHFR* gene was calculated by comparing the normalised expression values between different study groups. A fold-change value less than 1 indicate negative or down-regulation. A fold-change value greater than 1 indicates positive or up-regulation.

Statistical analysis

For qPCR, mean values of normalised expression ± standard deviation (SD) were obtained for each sample. Results were analysed by GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) using non-parametric Mann-Withney U-test comparing two groups based on normalised expression values for *MTHFR*. A value of $p<0.05$ was considered statistically significant.

MTHFR expression among RA patients with and without IHD and controls was first compared by analysis of covariance (ANCOVA), with adjustment for age, sex, and traditional CV risk factors.

Partial correlation between relative *MTHFR* expression and continuous variables, DAS (Disease Activity Score) 28 C-reactive protein (CRP) and DAS28 erythrocyte sedimentation rate (ESR), was performed after adjusting for age at the time of the study, sex, and classic CV risk factors via estimation of the Pearson partial correlation coefficient (r). Differences between *MTHFR* mRNA levels according to rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies status were analysed using ANCOVA adjusting for age, sex, and CV risk factors. These analyses were performed using Stata 12/SE (StataCorp, College Station, TX).

Results

Differences in MTHFR expression between RA patients and controls

MTHFR mRNA expression levels in peripheral blood samples were determined by qPCR. Gene-specific amplification was confirmed by a single peak

in the melting curve. This gene was expressed successfully in all samples and differences in the expression between patients with RA (n=26) and controls (n=10) were analysed.

A decreased expression of the *MTHFR* gene in patients compared to controls was found (fold change=0.85) (Fig. 1). In this regard, patients with RA had a mean *MTHFR* expression of 1.78±0.54, whereas controls had 2.10±0.23. This difference in expression was statistically significant (p=0.028).

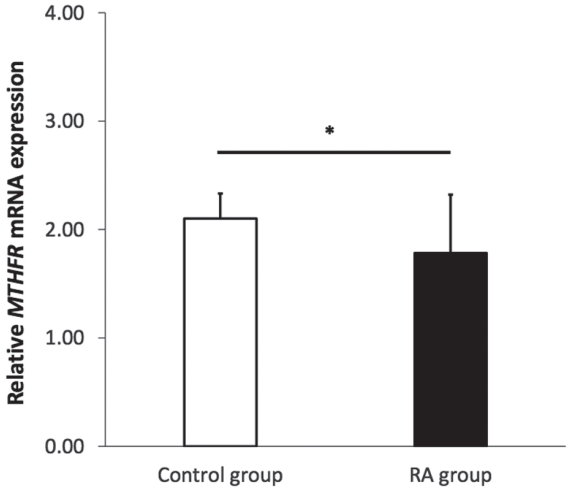

Fig. 1. Decreased *MTHFR* mRNA expression in the peripheral blood from patients with RA.
MTHFR expression was normalised to two housekeeping genes (beta-actin and GAPDH) and differential expression was analysed between control group (n=10) and RA group (n=26). Each bar represents mean value ± SD obtained for each sample in triplicate.
*indicates significance at p<0.05.

MTHFR expression and ischaemic heart disease

MTHFR gene expression in RA patients with IHD was significantly down-regulated when compared to controls (Fig. 2 and Table II). Although RA patients with IHD (n= 12) had less *MTHFR* expression (mean=1.65) than those patients without IHD (n=14; mean=1.90), the difference did not reach statistical significance. A slightly reduced *MTHFR* expression in RA patients without IHD when compared to controls was also found.

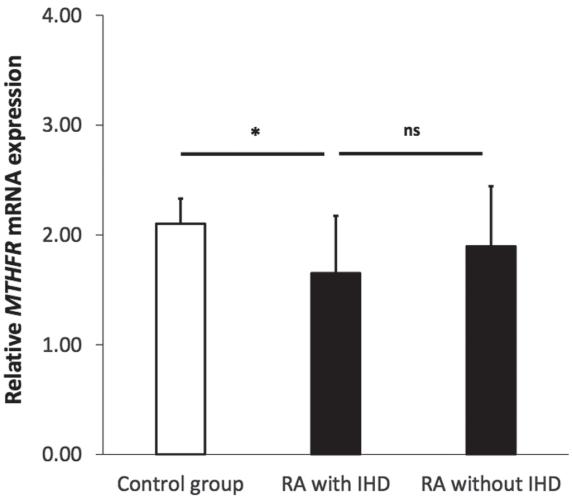

Fig. 2. *MTHFR* mRNA expression in RA patients stratified for the presence of IHD and controls.
Differential expression between RA patients stratified according to the presence of IHD (n=12) or absence of IHD (n=14) and controls. Each bar represents mean value ± SD obtained for each sample in triplicate.
*indicates significance at p<0.05.
ns indicates no significance (p>0.05).

Relationship of RA disease activity and RF and anti-CCP status with MTHFR expression

After an adjustment for age at time of study, sex, and classic CV risk factors, no statistically significant correlation between *MTHFR* levels of RA patients and disease activity variables (DAS28 CRP: r=-0.19; p=0.471 and DAS28 ESR: r=-0.16; p=0.528) was disclosed. Furthermore, no significant differences in the *MTHFR* expression according to RF and anti-CCP status were observed (data not shown).

Table II. *MTHFR* expression, fold change and p-values analysed between RA patients with IHD, RA patients without IHD and controls.

MTHFR expression	Expression	Fold change	p-value
RA patients with IHD compared to controls	↓	0.79	0.021
RA patients without IHD compared to controls	↓	0.90	0.121
RA patients with IHD compared to RA patients without IHD	↓	0.87	0.208

↓ indicates down-regulated expression.

Discussion

Although some studies focused on the potential implication of *MTHFR* gene polymorphisms in the susceptibility to RA or in the risk of subclinical atherosclerosis and CV events (35), there is no reported information on *MTHFR* gene expression in RA and specifically of CV disease in RA.

In line with the above, we previously described an association with endothelial dysfunction and CV events in patients with RA carrying the mutant C allele of the *MTHFR* A1298C gene polymorphism (32). In the present study, we have observed that the expression of the *MTHFR* gene is down-regulated in patients with RA.

Changes in the expression levels of *MTHFR* gene may be influenced by the use of MTX. Although 88.5% of our patients had been treated or were treated with MTX, all of them received folic acid supplementation (at least 5 mg weekly), what is known to reduce the risk of homocysteinaemia and to protect against CV risk (30, 36, 37).

Jia *et al*. disclosed a modest reduction of *MTHFR* gene expression in non-rheumatic individuals with coronary artery disease when compared with controls (38). In our study, RA patients with IHD had a significantly decreased expression of *MTHFR* gene compared to controls. We also observed a non-significantly reduced *MTHFR* gene expression in RA patients with IHD when compared to those patients without IHD. Because of that, it is possible that the reduction of *MTHFR* gene expression is associated with RA itself rather

than linked to any specific subgroup of RA patients. Nevertheless, it is plausible to think that more severe reductions of *MTHFR* gene expression in RA patients could account for an increased risk of IHD.

In conclusion, the results obtained in this study reveal for the first time a decreased *MTHFR* gene expression in peripheral blood from patients with RA, in particular in those with IHD. These results may support a potential implication of the transcriptional regulation of *MTHFR* in the pathogenesis of RA.

Acknowledgements

We wish to thank all the patients with RA and controls who participated to make this study possible.

We want to specially thank Jesús González Vela and Patricia Fuentevilla for their technical assistance.

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