Methylenetetrahydrofolate reductase polymorphisms and methotrexate: no association with response to therapy nor with drug-related adverse events in an Italian population of rheumatic patients

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ABSTRACT

Objective. *MTHFR is an enzyme involved in the folate pathway. It has been suggested that common polymorphisms in its gene (C677T and A1298C) could be related to different methotrexate (MTX) response and toxicity in rheumatoid arthritis (RA) patients. Agreement has not been found yet and there is no data on rheumatic Italian patients. The aim of this study is to determine if a genetic screening can help in planning the treatment in these patients.*

Methods. *We enrolled 84 Northern Italian patients affected by RA (n=79), psoriatic arthritis (n=4) and ankylosing spondylitis (n=1), who received MTX. Subjects who achieved at least ACR20 response in 6 months and maintained it during the following 6 months were defined as "responders"; those who did not obtain a disease control after 6 months of MTX were classified as "non responders". Patients who experienced MTX adverse events were defined "with toxicity", those who did not, as "without toxicity". Genotypes were determined by polymerase chain reaction.*

Results. *Genotype frequency was consistent with that reported in a healthy population from Italy. We did not find any statistically significant difference in genotype/allele distribution between the groups. In patients receiving folic acid supplementation MTX toxicity was recorded only in 18 cases (24%), while all the 8 patients not receiving it experienced MTX adverse events (p=0.00).*

Conclusion. *In our study we did not find any association between MTHFR genotype/allele and MTX response or toxicity. At the moment there is not sufficient evidence for MTHFR screening in patients who are candidate for MTX.*

Introduction

Methotrexate (MTX), a folate analogue, is the most used disease modifying antirheumatic drug for rheumatoid arthritis (RA).

Despite all its advantages, only 50% of patients has a good clinical response and up to 30% discontinues it for adverse events (ADEs) (1). Single nucleotide polymorphisms (SNPs), already studied in RA to investigate the susceptibility to disease (2) or to cardiovascular events (3), seem to play an important role in this variability.

Two common SNPs have been described for methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in the folate pathway: C677T (4) (cytosine to thymine substitution in nucleotide 677), that leads to a MTHFR variant with reduced activity, and A1298C (5) (adenine to cytosine substitution), that produces a milder reduction. It is not unreasonable to assume that genetic reduction of MTHFR activity may enhance folate metabolism inhibition induced by MTX. Several studies have suggested that 677 and 1298 MTHFR genotypes may be related to MTX efficacy or toxicity in RA patients, but agreement has not been reached yet and the role of these SNPs in Italian patients with rheumatic diseases has not been investigated.

The aims of this study are: to assess the prevalence of 677 and 1298 MTHFR SNPs among Italian rheumatic patients, to search for an association between particular genotypes/alleles and MTX efficacy/toxicity and to investigate the role of folic acid supplementation. The objective is to determine if a genetic screening of MTHFR can help in planning the most appropriate treatment in these patients.

Materials and methods

Patients

We retrospectively selected 105 native Italian patients regularly followed in our centre and treated with MTX. Basal disease activity, according to SDAI (6), was retrospectively calculated on the basis of parameters collected in clinical records.

We defined *responders* as RA patients who achieved at least an ACR20 response (7) during the first 6 months and maintained it during the following 6 ones. We excluded cases in which the response was associated to treatment modification with other drugs.

Rheumatoid arthritis patients who did not obtain a disease control after 6 months of MTX treatment were classified as *non responders*.

For toxicity evaluation we included,

in addition to RA patients, ankylosing spondylitis (AS) and psoriatic arthritis (PsA) patients. We considered ADEs occurred during the treatment after an adequate period of time, consistent with those known in the literature, improving or clearing up with MTX reduction or discontinuation, relapsing with MTX reintroduction and such as to require a therapy modification. ADEs were defined as follows:

General-gastrointestinal:
Nausea, anorexia, vomiting, diarrhea, abdominal pain, weight loss, malaise, fatigue, intolerance.
Hepatic:
Alanine aminotransferase values equal or higher than 3 times the upper limit of normal levels in absence of previous enzyme elevation, concomitant hepatotoxic drugs, positive viral hepatitis markers, alcohol consumption.
Cutaneous and mucosal:
Alopecia and mucosal ulcerations.
Neurological:
Headache, dizziness or mood alteration.
Hematological:
Leucopenia, thrombocytopenia or megaloblastic anemia.
Pulmonary:
Dyspnea (with or without dry cough, fever and malaise) associated with a new interstitial pneumonitis at x-ray, in absence of any demonstrable infectious etiology.
Subjects who never experienced MTX ADEs were considered without toxicity.
This study was approved by the Spedali Civili di Brescia Ethics Committee.

Genotype determination
A peripheral blood sample in EDTA (2.5 ml) was obtained from enrolled patients. DNA was extracted using the High Pure PCR Template Preparation Kit (Roche). MTHFR 677 and 1298 genotypes were determined by Light Cycler (Roche) real time polymerase chain reaction (PCR) (8).
The primers sequences were:
5'TGAAGGAGAAGGTGTCTGCGGGA 3' (forward)
3'AGGACGGTGCGGTGAGAGTG 5' (reverse)
for MTHFR 677 and:
5'CTTTGGGGAGCTGAAGGACTACTAC 3'(forward)
3'CACTTTGTGACCATTCCGGTTTG 5' (reverse)
for MTHFR 1298.

Statistical analysis
Quantitative data were expressed as the mean (± standard deviation) or median, qualitative ones as frequencies and percentages. Differences in variables were evaluated using Chi-square or Fisher's exact test (nominal) and Student's *t*-test (continuous). *P*-values less than 0.05 were considered significant.

Results

Among 105 patients matching criteria, 84 (all of Northern Italian origin) agreed to enter the study signing a written informed consent: 79 RA, 4 PsA and 1 SA. The weekly dose of MTX ranged between 7.5 and 20 mg/week. Most of our patients (92%) received folic acid supplementation (weekly dose equivalent to the MTX dose). Baseline characteristics are shown in Table I.
C677T SNP was present in most patients: 56% resulted heterozygote (CT) and 23% homozygote (TT). A1298C SNP was frequent, but wild-type genotype (AA=56%) was more frequent than mutated variants (AC=38%, CC=6%). The studied population was in Hardy-Weinberg equilibrium. We also studied genotype combination (677-1298) distribution; surprisingly three combinations were absent: homozygote-homozygote (O-O), heterozygote-homozygote (E-O) and homozygote-heterozygote (O-E).
First we compared frequencies obtained

Table I. Baseline characteristics of 84 genotyped patients.

Characteristics	Baseline value
Sex (female/male)	67/17
Age, mean ± SD	50 ± 13.9
Disease duration, median (range) months	1 (0-156)
Rheumatoid Factor positivity, %*	68
Simplified Disease Activity Index, mean ± SD*	32.27 ± 10.4

*Only for the 79 RA patients.

Table II. MTHFR genotype/allele frequency comparison between 84 patients and Italian healthy population (chi-square test, Yates' correction).

	Genotype/allele	All patients (n=84)		Healthy Italian population		χ^2	*p*
677	CC (wild-type)	18	21.43%	113	32%	3.13	0.07
	CT (heterozygote)	47	55.95%	166	47%	1.82	0.17
	TT (homozygote)	19	22.62%	74	21%	0.03	0.85
	C allele	83	49.40%	392	55%	1.81	0.17
	T allele	85	50.60%	314	45%	1.81	0.17
1298	AA (wild-type)	47	55.95%	114	56.44%	0.00	0.95
	AC (heterozygote)	32	38.10%	76	37.62%	0.00	0.95
	CC (homozygote)	5	5.95%	12	5.94%	0.07	0.78
	A allele	126	75%	304	75%	0.00	0.96
	C allele	42	25%	100	25%	0.00	0.96
677-1298	W-W	3	3.57%	9	4%	0.00	0.97
	W-E	10	11.90%	30	15%	0.25	0.61
	W-O	5	5.95%	13	7%	0.01	0.92
	E-W	25	29.76%	52	26%	0.25	0.61
	E-E	22	26.19%	56	28%	0.03	0.86
	E-O	0	0%	0	0%	–	–
	O-W	19	22.62%	40	20%	0.11	0.73
	O-E	0	0%	0	0%	–	–
	O-O	0	0%	0	0%	–	–

W: wild-type; E: heterozygote; O: homozygote.
Data on healthy Italian population come from (9) for 677, from (10) for 1298 and from (11) for 677-1298.

Table III. MTHFR genotype/allele frequency comparison between responders and non responders (chi-square test, Yates' correction or Fisher's exact test).

	Genotype/allele	Responders (n=37)		Non responders (n=32)		χ^2	*p*
677	CC (wild-type)	7	18.92%	6	18.75%	0.08	0.77
	CT (heterozygote)	19	51.35%	19	59.38%	0.10	0.75
	TT (homozygote)	11	29.73%	7	21.88%	0.22	0.64
	C allele	33	44.59%	31	48.44%	0.07	0.77
	T allele	41	55.41%	33	51.56%	0.07	0.77
1298	AA (wild-type)	21	56.76%	16	50.00%	0.10	0.74
	AC (heterozygote)	14	37.84%	14	43.75%	0.06	0.80
	CC (homozygote)	2	5.41%	2	6.25%	(Fisher)	0.38
	A allele	56	75.68%	46	71.88%	0.09	0.75
	C allele	18	24.32%	18	28.13%	0.09	0.75
677-1298	W-W	0	0.00%	2	6.25%	(Fisher)	0.21
	W-E	5	13.51%	2	6.25%	(Fisher)	0.20
	W-O	2	5.41%	2	6.25%	(Fisher)	0.38
	E-W	10	27.03%	7	21.88%	0.05	0.82
	E-E	9	24.32%	12	37.50%	0.85	0.35
	E-O	0	0.00%	0	0.00%	–	–
	O-W	11	29.73%	7	21.88%	0.22	0.64
	O-E	0	0.00%	0	0.00%	–	–
	O-O	0	0.00%	0	0.00%	–	–

W: wild-type; E: heterozygote; O: homozygote.

Table IV. MTHFR genotype/allele frequency comparison between patients with and without MTX toxicity (chi-square test, Yates' correction or Fisher's exact test).

	Genotype/allele	Patients with toxicity (n=26)		Patients without toxicity (n=68)		χ^2	*p*
677	CC (wild-type)	8	30.77%	10	17.24%	1.23	0.26
	CT (heterozygote)	13	50.00%	34	58.62%	0.25	0.61
	TT (homozygote)	5	19.23%	14	24.14%	0.05	0.82
	C allele	29	55.77%	54	46.55%	0.87	0.34
	T allele	23	44.23%	62	53.45%	0.87	0.34
1298	AA (wild-type)	17	65.38%	30	51.72%	0.86	0.35
	AC (heterozygote)	7	26.92%	25	43.10%	1.37	0.24
	CC (homozygote)	2	7.69%	3	5.17%	(Fisher)	0.32
	A allele	41	78.85%	85	73.28%	0.33	0.56
	C allele	11	21.15%	31	26.72%	0.33	0.56
677-1298	W-W	2	7.69%	1	1.72%	(Fisher)	0.19
	W-E	4	15.38%	6	10.34%	(Fisher)	0.21
	W-O	2	7.69%	3	5.17%	(Fisher)	0.32
	E-W	10	38.46%	15	25.86%	0.83	0.36
	E-E	3	11.54%	19	32.76%	3.16	0.07
	E-O	0	0.00%	0	0.00%	–	–
	O-W	5	19.23%	14	24.14%	0.05	0.82
	O-E	0	0.00%	0	0.00%	–	–
	O-O	0	0.00%	0	0.00%	–	–

W: wild-type; E: heterozygote; O: homozygote.

from our sample with those observed in the healthy population. We used as controls data on MTHFR 677 (9), 1298 (10) and their association (11) in healthy Northern Italian subjects. No significant differences in both allele and genotype distribution were found (Table II). The same results were obtained considering only RA patients rather than all patients.

To study the relationship between MTHFR genotypes/alleles and MTX efficacy we made a comparison between their distribution in responders and non responders (Table III). No statistically significant difference emerged. Basal disease activity according to SDAI was not significantly different in responders (32.4±12) and non responders (29.2±12).

Twenty-six subjects experienced MTX toxicity at a very variable time (range: 1-74 months). 76% had general-gastric toxicity, 23% hepatic, 11% cutaneous-mucosal, 15% neurological, 3% hematological and 3% pulmonary. We did not find any statistically significant difference in genotype/allele distribution between patients with and without ADEs (Table IV).

All the 8 subjects who were not taking folic acid experienced MTX toxicity. In contrast, patients who were receiving folic acid were in most cases protected against ADEs (toxicity only in 24% of cases). The difference is statistically significant (p=0.00).

Discussion

Several studies, mostly retrospective, have been conducted since Van Ede *et al*. (12) raised the possibility of a pharmacogenetic approach to MTX treatment considering MTHFR SNPs. Globally, these works have not reached a definite conclusion as to the clinical relevance of such genetic variations.

In our study genotype/allele distribution was found different from that reported in rheumatic patients from other countries. This discordance should be attributed to ethnic inhomogeneity within the populations considered (13). Comparison with data on this Italian healthy population showed that rheumatic patients have the same genotype/allele distribution. In particular we observed, consistently with previous studies on Italian people (9), a high frequency of C677T SNP. Moreover, we found among carriers a considerable group (23%) of homozygotes (TT). Our findings are in line with a previous study (13) suggesting that MTHFR genotype/allele frequencies are similar in patients and controls of the same racial ethnic group. This is in contrast with a recent observation (2) where an excess of homozygotes for 1298 (CC) among RA patients was described.

Furthermore, we observed the absence of 3 genotypic combinations. In a previous study (11) the same combinations

were found absent in the control group (healthy Italian subjects). Since both SNPs are associated with the reduction of enzyme activity, O-Os were arguably removed during evolution (14) because the enzyme had too low an activity to survive. It is possible to assume that also O-Es and E-Os were negatively selected from the Italian population.

C677T and A1298C SNP analysis in our study did not reveal any association between a genotype or an allele and MTX efficacy or toxicity.

Our study confirmed that folic acid supplementation can provide a protection against MTX toxicity. A study on MTHFR from *Escherichia coli* (15) also demonstrated that folic acid counteracts the negative effect of MTHFR C677T SNP. The variability in results of previous studies may be partially due to a different folate intake. Further studies could clarify whether folic acid is able to restore MTHFR activity in human cells.

Due to the retrospective nature and to the focus on a specific population, our study lacks proper randomization and a significant number of subjects. However, this is, to our knowledge, the first study of MTHFR SNPs/MTX correlation in Italian rheumatic patients. Our analysis encompassed subjects with diseases different from RA, since we expect that a larger range of rheumatic patients may benefit from a pharmacogenetic approach to MTX treatment.

Compared to our work, most studies did not define accurate criteria to identify MTX toxicity, but simply registered ADEs compatible with MTX. These criteria, we submit, are crucial to avoid correlating MTHFR genotypes to events not directly linked to the drug.

Conclusions

Although expected on theoretical grounds, we did not find any association between MTHFR genotypes/alleles and MTX response. State-of-the-art results do not provide convincing evidence for the need of MTHFR screening in patients who are candidate for MTX. Waiting for further pharmacogenetic results, folic acid supplementation remains the most effective and inexpensive way to protect patients against MTX ADEs.

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