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Methionine synthase polymorphism A2756G is associated with susceptibility for thromboembolic events and altered B vitamin / thiol metabolism

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Background and Objectives. Vitamin B12 dependent methionine synthase (MS) regulates de novo production of methionine from homocysteine (Hcy). Since moderate elevations in Hcy are considered vasculotoxic, we examined a common variant (A2756G-MS) of the gene coding for this enzyme as a risk for thromboembolism.

Design and Methods. We investigated A2756G-MS and folate / thiol status in 51 individuals who had experienced a thromboembolic event (TE) and 95 subjects being treated for non-thromboembolic (NTE) vascular problems.

Results. The prevalence of the mutant G allele was lower in TE subjects than in controls, indicating a protective role for this base substitution (OR 0.39; 95%CI 0.20-0.78; p=0.010). Consistent with an advantage conferred by this allele, heterozygotes had generally lower levels of Hcy and glutathione (GSH), and higher levels of B-vitamins than wildtypes. The OR for the wildtype having an increased risk for TE was 2.32 (95%CI 1.06-5.08). Additionally, as might be predicted, TE-wildtypes had elevated GSH levels compared to corresponding NTE-wildtypes (p=0.004) - a likely response to oxidative stress. NTE subjects showed a dramatic reduction in Hcy between wildtype and heterozygote (p=0.017), and again between recessive and heterozygote genotypes (p=0.002). The same pattern, although not significant, occurred in TE subjects. The similarity in Hcy between clinical groups for each genotype raises questions on the etiological role of Hcy in TE. The functional relationship between enzyme variant and its B_{12}-cofactor may be of more interest, since the polymorphic site occurs near the B_{12}-binding domain, and our results indicate wildtype-TE subjects have a much lower level of vitamin B_{12} than heterozygote-TE subjects (p=0.0019). This effect is attenuated in NTE subjects.

Interpretation and Conclusions. A2756G-MS may protect against a thromboembolic event. The role of Hcy remains unclear.

Key words: folate, homocysteine, thromboembolism, A2756G methionine synthase, polymorphism.

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Vitamin B_{12} dependent methionine synthase (MS) is an important folate enzyme that utilizes the methyl group of 5-methyl-H_4folate to methylate homocysteine (Hcy) in the *de novo* production of methionine and s-adenosylmethionine. This reaction also regenerates H_4folate for the many biosynthetic intracellular single-carbon (1C) transfer reactions that depend upon the folate cycles.

The gene coding for MS has a common polymorphic form (A2756G, D919G). This base transition, converting an aspartic acid into glycine, occurs near the crucial vitamin B_{12} binding site, and therefore might influence the enzymes secondary structure with possible functional consequences. In a recent case-control study A2756G MS was shown to be associated with a tiered reduction in Hcy and increase in folate within controls but not myocardial infarction cases.[1] This finding is broadly the opposite of that found in the common C677T methylenetetrahydrofolate reductase polymorphism (C677T MTHFR). In C677T MTHFR an association is considered to exist between elevated plasma homocysteine (Hcy), and both TT C677T MTHFR genotype and vascular disease, although any association between the TT genotype and vascular disease is less clearly defined and remains controversial.[2]

MS plays a strategic role in regulating Hcy. Even a moderate accumulation in Hcy is generally undesirable since a build up of reactive oxygen species during the auto-oxidation of Hcy elicits endothelial damage. This is particularly true where the capacity of endogenous antioxidants is exceeded. The result of this is impaired endothelial function with perturbations in vasoregulation and antithrombotic mechanisms that promote platelet adhesion and thrombus production.[3]

Since A2756G MS seems to attenuate Hcy levels which are potentially atherothrombogenic,[1] we examined MS genotype, B vitamin- and thiol metabolism in 51 cases who had experienced a thromboembolic (TE) event (i.e. deep vein thrombosis) and 95 control patients being treated for non-thromboembolic (NTE) vascular problems (i.e.

valve replacement). We present data on vitamin B_{12}, erythrocyte folate, plasma folate, Hcy, cysteine (Cys) and an index of transsulphuration that is indicative of oxidative stress - glutathione (GSH). Redox changes associated with oxidative stress are of interest since they may increase the flux of Hcy through the transsulphuration pathway to Cys and GSH via a regulatory role at MS and cystathionine-β-synthase. This, it is claimed, may be a self-correcting response to depleted GSH in cells facing oxidative challenge.[4] We were, therefore, particularly interested to see whether A2756G MS does reduce Hcy and offer protection against TE events, and in so doing, whether it is associated with lower GSH levels.

Design and Methods

Subjects

To clarify the association between A2756G MS G allele frequency and risk of a TE event, we examined 146 patients attending the local anticoagulant clinic. Samples were collected over 9 months and separated according to clinical history into those from individuals who had experienced a TE event (i.e. deep vein thrombosis) or those from patients who were being treated for NTE vascular problems such as valve replacement. These individuals were designated as case (n=51) or control (n=95) subjects, respectively. The median age and interquartile range (IQR) for TE and NTE subjects was 67.5 years (52-77) and 71 years (60-79), respectively. The difference in age between groups was not significant. TE subjects were 51% male, 49% female and NTE subjects 46% male, 54% female. Table 1 provides clinical data on each cohort.

Table 1. Clinical background for TE and NTE cohorts.

Thromboembolic event	
% Pulmonary embolism	36.5%
% Deep vein thrombosis	42%
% Other (ie thrombotic cerebrovascular accident)	21.5%
Non-thromboembolic event	
% Cardiac arrhythmia	62%
% Valve disease/repair/replacement	31%
% Other (ie peripheral vascular disease, transient ischemic attack, angina, myeloproliferative disease, dilated cardiomyopathy)	7%

Table 2. Number and frequency of alleles for A2756G MS in 51 thromboembolic and 95 non-thromboembolic subjects.

	A2756G MS allele number (frequency %)	
Subject group	A	G
Thromboembolic	90 (88.2)	12 (11.8)
Non-thromboembolic	142 (74.7)	48 (25.3)

Analysis

The assay for plasma thiols measures both oxidized and reduced forms. For example, total Hcy refers to homocysteine, homocystine, homocysteine-cysteine mixed disulphide and protein-bound forms of Hcy. The same holds true for analysis of total Cys and total GSH. Plasma total thiols (Hcy, Cys and GSH) were measured using isocratic HPLC following derivatization with the fluorogenic reagent SBDF.[5] Plasma and red cell folate along with vitamin B_{12} were measured using a paramagnetic-particle, chemiluminescent immunoassay (Access Immunoassay System, Beckman Instruments, Inc).

A2756G MS genotyping was based on the method of Van der Put *et al.*[6] Briefly, sense and antisense primers with the sequences 5'-GGT GTG TTC CCA GCT GTT AGA TG-3' and 5'-GAC ACT GAA GAC CTC TGA TTT GAA C-3' respectively, were used for PCR amplification. The amplicons were digested with the restriction enzyme *Hae*III to yield a 265 bp fragment for the wildtype (AA), 265, 180 and 85 bp fragments for the heterozygote (AG), and 180 and 85 bp fragments for the homozygous recessive genotype (GG).

Statistics

The Anderson-Darling test was used to ascertain normality. Significant differences for unpaired data were then established using either an independent two sample t- or Mann-Whitney test. The degree and significance of an allele as a risk factor for TE was calculated using the Odds ratio with associated confidence intervals. The *p* value was obtained using Yates' corrected χ^2 test.

Results

The frequency of AA, AG and GG genotypes was 78.4, 19.6 and 2.0%, respectively, for TE subjects and 61.1, 27.4 and 11.6%, respectively, for NTE individuals. Table 2 gives both allele number and allele frequency.

An Odds ratio indicates that the G allele may afford significant protection against TE: OR = 0.39, 95% CI 0.20-0.78. χ^2 gives a *p* value of 0.0065 (Yates' corrected *p* value = 0.01). Carriage of the G

Table 3. Median and interquartile range values for B vitamin and total thiol levels in patients with each of the A2756G MS genotypes for 51 thromboembolic and 95 non-thromboembolic subjects. Superscript numerals indicate a significant difference either within or between clinical groups: $p = 0.0173^1$, 0.0020^2, 0.0019^3 and 0.0035^4.

	AA 2756 MS		AG 2756 MS		GG 2756 MS		All	
Metabolite	TE	NTE	TE	NTE	TE	NTE	TE	NTE
Hcy (μMol/L)	9.6 (8.0-14.5)	10.6[1] (7.9-13.1)	8.9 (6.8-9.7)	8.4[1,2] (7.6-9.9)	14.7 (na)	11.2[2] (10.1-13.0)	9.4 (7.6-13.6)	9.9 (7.9-12.9)
Cys (μMol/L)	236 (204-263)	257 (224-287)	196 (185-239)	249 (232-277)	386 (na)	262 (224-308)	234 (198-265)	255 (224-285)
GSH (μMol/L)	4.8[4] (3.9-5.8)	3.8[4] (3.0-4.5)	3.8 (2.9-7.3)	3.4 (2.5-4.9)	4.9 (na)	3.0 (2.2-3.7)	4.8 (3.6-5.8)	3.6 (2.9-4.5)
Vitamin B_{12} (ng/mL)	336[3] (219-428)	342 (266-453)	544[3] (375-713)	389 (286-549)	524 (na)	415 (338-543)	363 (237-520)	359 (270-492)
Serum folate (ng/mL)	5.2 (3.8-8.3)	6.1 (4.2-8.9)	7.9 (4.7-10.9)	6.9 (5.3-11.7)	13.3 (na)	5.6 (4.2-8.3)	5.4 (3.9-8.9)	6.2 (4.4-8.9)
RBC folate (ng/mL)	177 (104-207)	183 (140-232)	180 (134-262)	198 (116-299)	165 (na)	165 (110-238)	177 (110-214)	189 (134-256)

allele (AG + GG versus AA) also seemed to afford protection against TE: OR = 0.43, 95% CI 0.20-0.94. χ^2 gave a p value of 0.0331 (Yates' corrected p value = 0.05). When each genotype was individually compared to the wildtype, an OR consistent with a protective effect against TE was still observed, although significance was not quite achieved (OR = 0.13 and 0.56 for AA versus GG and AA versus AG, respectively).

Table 3 lists the median and interquartile range (IQR) for blood B vitamin and thiol levels between patients with the various MS genotypes in each clinical group, and indicates any statistical differences (both within and between clinical groups). A comparison of all TE and NTE subjects, independent of MS genotype, showed that only two parameters differed significantly: GSH was higher in TE individuals than in NTE ones ($p = 0.0008$), while its precursor Cys was lower in TE subjects than in those with NTE subjects ($p = 0.0146$). When examined on a genotype-dependent basis, three major statistical differences were detected, which in the first two cases, support the view that A2756G MS may exhibit a heterozygote advantage:

- significantly lower Hcy in NTE-AG patients compared to in NTE-AA ones; $p = 0.0173$ (also significantly lower Hcy in NTE-AG patients compared to in NTE-GG ones; $p = 0.002$);
- significantly higher vitamin B_{12} in TE-AG patients compared to in TE-AA ones; $p = 0.0019$;
- significantly higher GSH in TE-AA patients compared to in NTE-AA ones; $p = 0.0035$.

Although significance was not always attained, the overall trend for all measured parameters in Table 3 indicates that A2756G MS exhibits a heterozygote advantage. This is deduced because in both clinical groups the change between AA and AG genotypes was associated with an increase in serum folate, RBC folate and vitamin B_{12}, and a concomitant decrease in Hcy, Cys and GSH. In other words, the heterozygote equilibrium would seem to be moving in the direction of increased MS turnover as opposed to Hcy build up or transsulphuration.

It is also worth mentioning that Cys levels in TE patients with AA and AG genotypes were lower than Cys levels in the corresponding NTE patients with AA and AG genotypes. This reduction approached significance in both cases - $p = 0.0725$ and 0.0582, respectively.

Discussion

The central role of folate metabolism in nucleotide and methionine biosynthesis means that common single nucleotide polymorphisms (SNPs) that influence 1C transfers can act as both a risk factor and defence against disease. For instance, C677T MTHFR is a risk factor for neural tube[7] and other mid-line defects,[8] Down's syndrome[9] and complications of pregnancy such as pre-eclampsia[10,11] and recurrent early pregnancy loss.[12] However, it can also, under certain circumstances, protect against colon cancer[13] and acute lymphoblastic leukemia.[14] The most likely explanation for this dichotomy of effect is that C677T MTHFR increases levels of 5,10-methylene-H_4folate, a critical coenzyme required for nucleotide biosynthesis (Figure 1). Under good folate nutrition, an accumulation of 5,10-methylene-H_4folate might actually protect against cancer.[13] However, under poor folate nutritional status, this same SNP might compromise cellular levels of 5,10-methylene-H_4folate and augment uracil misincorporation, a process that leads to DNA instability and possibly carcinogenesis. C677T MTHFR has other likely effects on 1C metabolism, but these are generally outside the scope of this report.[15]

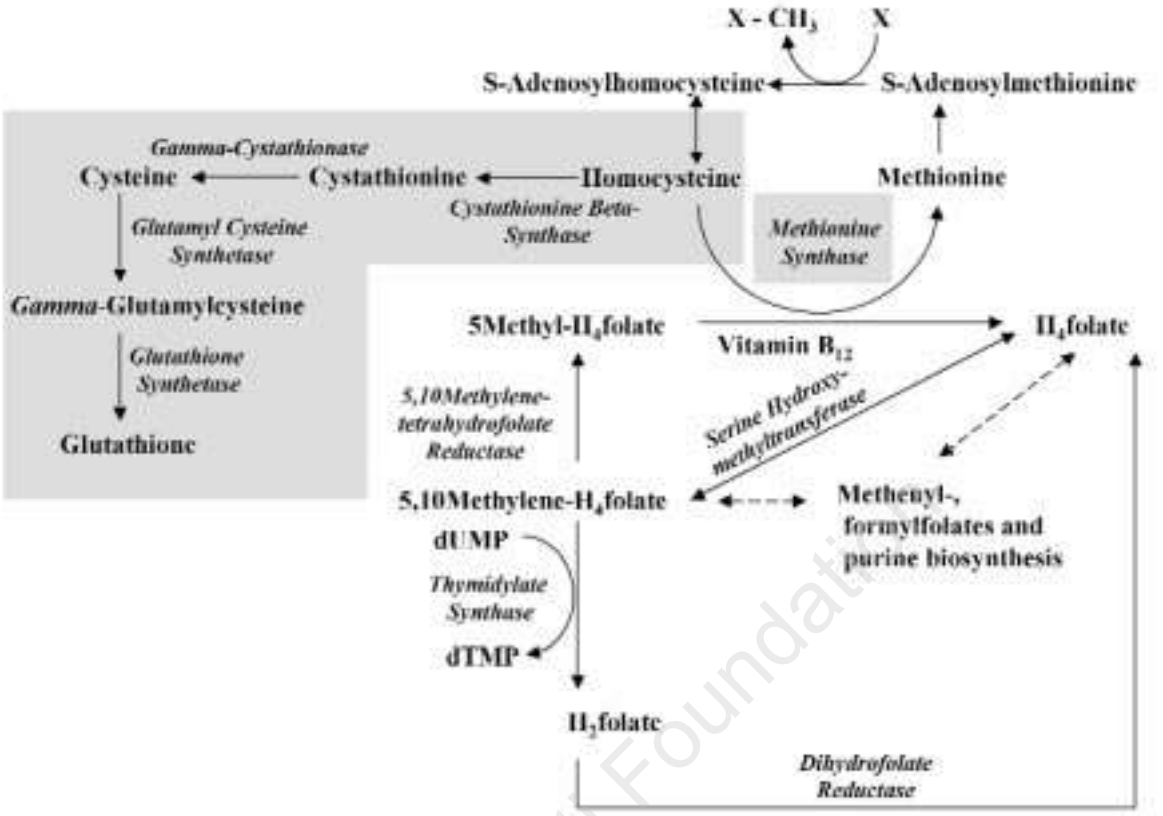

Figure 1. The central role of MS in the transmethylation and transsulphuration (grey box) of Hcy.

In the present study it appears that heterozygosity for A2756G MS is associated with the lowest Hcy (and Cys) levels as well as diminished GSH and enhanced vitamin B_{12} and folate status. These are all positive indices and are consistent with the significant finding that carriage of the G allele offers reduced risk of a thromboembolic event (OR 0.39). Thus, A2756G MS, like C677T MTHFR, may, under certain conditions, be another folate SNP with possible health benefits.

It is interesting to consider the role of MS in Hcy metabolism, and in particular the balance between Hcy remethylation, and transsulphuration to Cys and on to GSH (Figure 1). The data presented here are consistent with both clinical groups benefiting from heterozygosity for this SNP. Of course, it is equally clear that among individuals who do not carry a mutant allele, there is an increased risk of TE (OR = 2.32 95% CI 1.06-5.08). Indeed, compared to NTE-wildtypes, TE-wildtypes exhibit a significantly elevated level of GSH and a lower Cys (approaching significance). Hcy levels do not differ between these two categories. This would seem to suggest that although A2756G MS is capable of altering the turnover of Hcy at this enzyme locus, the effect does not obviously differ between clinical groups. This is despite more Cys apparently being used to enhance GSH levels in TE subjects than in NTE subjects. Therefore, the negative influence of carrying no mutant MS allele in TE subjects is most likely increasing GSH production as a mechanism to attenuate oxidative stress. However, while Hcy levels are generally higher in individuals with the wildtype MS enzyme than in heterozygotes, no significant difference exists between TE and NTE subjects. This suggests that Hcy may, in fact, not be a clinical factor in the causation of TE in these subjects. A similar conclusion regarding the vasculotoxic role of Hcy has been drawn by other researchers studying a link between vascular problems and the closely related polymorphic A66G methionine synthase reductase (MSR) gene.[16]

Although in general terms, mild hyperhomocys-

teinemia is now a well-established risk factor for a TE event, this was not evident from the present study. Others have also examined A2756G MS as a risk factor for TE: Salomon *et al.*[17] concluded that A2756G MS was not a statistically significant risk factor for idiopathic venous thromboembolism. Despite this, and in accord with our data, Tsai *et al.*[18] have recently shown that the A2756G transition is associated with lower Hcy levels, while Hyndman *et al.*[19] reported that heterozygosity for A2756G MS increases red cell folate and reduces secondary vascular events. The designs of these various studies differ. Our own findings may in part be a reflection of our control population, who are in themselves, a cohort of patients with vascular problems. Despite this, our study contributes further preliminary data indicating that the common A2756G polymorphism of MS may be an etiological factor in TE. However, like previous studies that implicate this SNP as a factor in myocardial infarction,[1] larger studies would be desirable. Such studies might also be able to detect exactly how the biological function of MS is altered in this SNP. At this stage it is interesting to speculate that it may be linked to vitamin B_{12} metabolism in some way since the mutation occurs close to the B_{12} binding domain, and both clinical groups exhibit increasing plasma vitamin B_{12} levels with increasing carriage of the mutant allele.

In fact, in the *at risk* TE patients with a wildtype genotype, plasma vitamin B_{12} levels are significantly lower than in the TE-heterozygote genotype patients ($p = 0.0019$).

As interesting as these findings are, considerable work is required for a full elucidation of the complexity of all the possible interactions and clinical consequences involved. In particular, the potential existence of polymorphisms in the 3' untranslated sequence of the MS gene requires further study, since such variants might influence the MS message and levels of the enzyme. The interaction (linkage disequilibrium) between such putative mutations, A2756G MS and three silent MS mutations (A2053T, A2127G and A3144G), as well as with related SNPs such as A66G MSR will, most likely, be of great interest in this particular area of clinical research in the future.

In summary, this study shows how folate, thiol and vitamin B_{12} metabolism are intimately linked at the level of this important genetic locus. It also illustrates that folate biochemistry (particularly genetic variation and nutrition) is a likely factor in vascular disease.

Contributions and Acknowledgments

ZY: planning, analysis, interpretation, formulation and critical revision of paper, approval of final manuscript; ML: conception and design of project, interpretation and analysis of data, formulation and critical revision of paper, particularly for intellectual content, approving final version of manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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PEER REVIEW OUTCOMES

Manuscript processing

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What is already known on this topic

It has been indicated that high plasma levels of homocysteine increase the risk of thromboembolic events. Methionine synthase polymorphism A2756G seems to attenuate homocysteine plasma levels.

What this study adds

The authors investigate the prevalence of this polymorphism and vitamin B_{12}/thiol metabolism in patients who had experienced a thromboembolic episode and controls.

Potential implications for clinical practice

The results suggest that A2756G methionine synthase polymorphism may protect against thromboembolic events. However, the role that homocysteine plasma levels play in this association is not clear.

Vicente Vicente, Deputy Editor