Effect of common polymorphisms in folate uptake and metabolism genes on frequency of micronucleated lymphocytes in a South Australian cohort

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Keywords: Folate Lymphocytes Micronuclei Single nucleotide polymorphisms

1. Introduction

Folate deficiency is associated with increased DNA strand breaks, chromosomal damage and micronucleus formation in lymphocytes, however, whether these effects may also depend on inherited or acquired defects in folate uptake and metabolism remains uncertain [1,2]. Genetic deficiencies in folate and methionine pathways have been linked with various adverse health conditions ranging from birth defects, pregnancy complications, cardiovascular disease, certain cancers and cognitive dysfunction [3–5] and these effects may be caused by increased chromosomal damage [6]. It is therefore important to determine the impact of common polymorphisms in genes that code for proteins and enzymes required for folate uptake, transport and metabolism on validated biomarkers of genome damage such as micronuclear frequency which has been linked prospectively with elevated risk for cancer and cardiovascular disease [7,8].

The key pathways and genes involved in folate uptake and metabolism are described in Fig. 1. The glutamate carboxypeptidase II (GCP II) gene, also known as the folate hydrolase gene, codes for the enzyme folylpoly-γ-glutamate carboxypeptidase which is anchored to the intestinal apical brush border membrane and facilitates the digestion (or breakdown) of dietary folates (a mixture of polyglutamated folates) to monoglutamyl folates which are absorbed into the blood stream after reduction and methylation [9]; in the case of the synthetic form of folate (i.e. folic acid), found in supplements, reduction and methylation to 5-methyltetrahydrofolate (5-MeTHF) mainly occurs in the liver [10]. Monoglutamate 5-MeTHF is the predominant circulatory form of folate which is transported to peripheral tissues through the blood stream and subsequently transported into the cells by the reduced folate carrier (RFC), a transmembrane glycoprotein [11]. Methionine synthase (MTR) catalyzes remethylation of homocysteine (Hcy) to methionine using methyl groups from 5-MeTHF and methionine synthase (MTR) catalyzes remethylation of homocysteine (Hcy) to methionine using methyl groups from 5-MeTHF.
MTRR (A66G, C524T), micronucleus expression in lymphocytes [15–18]. SNPs in these genes involved in the Hcy remethylation pathway may contribute to the presence of its cofactor vitamin B12 and homocysteine, which maintains the methionine synthase-bound B12 in its fully reduced active state [14]. Tetrahydrofolate (THF), which is generated from the MTR reaction, is re-methylated to 5,10-methyltetrahydrofolate (5,10-MeTHF) whilst thymidylate synthase (TS) catalyzes the conversion of deoxyuridine monophosphate to thymidine monophosphate using 5,10-MeTHF as a methyl donor. Methylenetetrahydrofolate reductase (MTHFR) plays a key role in folate metabolism by irreversibly converting 5,10-MeTHF back to the primary circulating form of folate 5-MeTHF (Fig. 1). Previous studies in European and Asian cohorts have suggested that common polymorphisms in some of the genes (e.g. MTHFR C677T, MTR A66G) discussed above may have an impact on micronuclear expression in lymphocytes [15–18]. SNPs in MTHFR (C677T; A1298C), MTR (A2756G) and MTRR (A66G) genes have been linked with elevated Hcy concentrations in some populations and Hcy has been shown to be positively correlated with lymphocyte micronuclear frequency [19–24]. These studies suggest that genetic variants of these genes involved in the Hcy remethylation pathway might act as predisposing factors contributing to elevated micronuclear frequency.

The aim of the present study was to examine the strength of the association between MTHFR (C677T, A1298C), MTR (A2756G), MTRR (A66G, C524T), CEP2 (C1561T), RFC (G80A) and TS (tandem repeats, 6bp deletion in 3′-UTR region) gene SNPs and frequency of lymphocytes with micronuclei in a healthy Australian cohort. This information, which was not available previously, is important because (a) one cannot assume that genotype-genome damage relationships observed in other countries is applicable to Australians given the different nutritional and environmental background and (b) it informs future studies on the genetic variables that need to be accounted for when designing and interpreting data from investigations aimed at determining the effect of environmental, dietary and life-style factors on DNA damage measured using the cytokinesis-block micronucleus (CBMN) assay. Furthermore, this study is distinguished from previous investigations by also examining genes involved in folate uptake from the gut (e.g. GCPII) and into cells (e.g. RFC).

2. Materials and methods

2.1. Subjects

In the present study, 164 healthy non-smoking individuals (94 males and 70 females) of different age ranging from 18 to 73 years were recruited following advertisements in the local newspaper after their informed consent was given. The study was approved by the CSIRO Human Experimentation Ethics Committee. Their mean (±SEM) plasma folate (nmol/L), red cell folate (nmol/L), vitamin B12 (pmol/L) and homocysteine (μmol/L), was 13.07 ± 0.59 nmol/L, 479.6 ± 16.33 nmol/L, 315.9 ± 10.24 pmol/L and 9.05 ± 0.19 μmol/L respectively, which is within the normal clinical range for these parameters.

2.2. Blood collection and cytokinesis-block micronucleus (CBMN) assay

Whole blood (10 mL) was collected into a lithium heparin vacutainer tube. Lymphocytes were isolated from the heparinised blood samples by diluting 1:2 with Hank’s balanced salt solution (HBSS, Sigma, Australia) and using Ficoll Hypaque gradients (Pharmacia Biotech, Uppsala, Sweden). The isolated lymphocytes were washed twice in HBSS before estimating cell concentration using Coulter Counter (Beckman Coulter Model ZBI, Setting: Attenuation 1; Threshold: 8; Aperture: 1/4; Manometer: 0.5). The CBMN assay was performed according to a standard protocol as described previously for isolated lymphocyte cultures [25]. Cells were cultured in 1 mL volumes at a concentration of 1 × 10⁶ cells/mL in RPMI-1640 (Trace, Australia) containing 10% FCS (Trace, Australia), and 2 mM l-glutamine (Sigma, Australia). Each culture was set up in duplicate and 10 μL/L of phytohaemagglutinin (PHA; Murex Biotech, Kent, England) was added to each culture and then incubated at 37 °C for forty-four hours after PHA stimulation. 4.5 μg/mL of Cyclophalasin B (Sigma, Australia) was added to the cultures to accumulate cells that had completed one nuclear division at the binucleate stage [25]. Cells were harvested between 68 and 72h after PHA stimulation. Cells were transferred to slides using a cyto-centrifuge (Shandon Cytoformer North Southam Products, Cheshire, UK). Slides were air-dried for 10 min before being fixed and stained using Diff Quik (Lab Aids, Australia). Each slide was scored for the number of binucleated cells (BNCs) containing one or more micronuclei (BN-MN) in 1000 binucleated cells. Criteria for identifying binucleated cells and micronuclei within binucleated cells were as described previously [25].

2.3. Genetic analyses

DNA was extracted from blood samples using standard techniques. Nine SNPs in six folate pathway genes were examined either by PCR-RFLP or ARMS-PCR method. Table 1 lists the polymorphisms examined in this study and reaction conditions employed for amplification including the primer sequences. Some of these loci were not amplified from every subject due to problems with DNA quality and/or PCR amplification. SNPs in MTHFR (C577T, A1298C) were analyzed by PCR and allele specific primer restriction digestion with Hifil and Mbol respectively and as described previously [26,27]. SNPs in exon 13 of GCPII C516T [28], exon 2 of RFC G80A [29], exon 26 of MTR A2756G [13], MTRR A66G [30] were studied as described previously. The MTR (C524T) polymorphism was determined using allele-specific primer pairs-PCR (amplification refractory mutation system; ARMS-PCR) method [31]. Polymorphisms in thymidylate synthase gene (tandem repeats in 5 regulatory region and 6bp deletion at 1494 in 3′-UTR region) were analysed as described previously [32,33]. The digested/undigested PCR products (10 μL) were run onto 2% agarose gel, except for MTHFR A1298C which was run onto 2.5% MetaPhor® agarose, stained with ethidium bromide and visualized under UV illumination.

2.4. Statistical analysis

Allelic and genotype frequencies for each SNP were calculated from the genotyping data and Hardy–Weinberg equilibrium was determined using an online available tool (http://www.genes.org.uk/software/hardy-weinberg.shtml). BN-MN frequency data were adjusted for the effect of age using a previously described formula [19], BN-MN25.5y = (25.5 – X)×M, where X = actual age in years, 5° slope of regression line for relationship between age and BN-MN cell frequency, M = actual BN-MN cell frequency measured and BN-MN25.5y = the BN-MN cell frequency adjusted to the value expected at age 25.5 years. Adjustment for gender effect on BN-MN cell frequency was performed using the ratio of the mean BN-MN cell frequency in males and females as the correction factor. We performed a power calculation on a model assuming approximately 80 subjects per carrier or non-carrier group (given a total N = 160) and detection of an effect size between 1.8 to 2.9 age- and gender-adjusted BN-MN per 1000 BN cells. We had decided on this effect size because this level of BN-MN is induced by an acute dose of between 5 cGy and 10 cGy (equivalent to 50 milleSv/urets and 100 milleSv/urets) of low LET ionising radiation (e.g. X-rays) [34] which is biologically meaningful and substantial given that this acute radiation dose range is associated with a significant and substantial increased cancer risk of approximately 4% [35]. Based on the standard deviation for age- and gender-adjusted BN-MN of 6.5 for the whole group, the study had sufficient power to detect a difference between groups of 2.9, 2.6, 2.3, 2.0 and 1.8 at 80%, 70%, 60%, 50% and 40% power at P < 0.05 respectively. Comparison of BN-MN frequency between two groups was performed using both an unpaired t-test on log10-transformed data and the Mann-Whitney U test on untransformed data because the distribution of BN-MN frequency often tended to be Gaussian in one group but not in the other. For comparison of data in more than two groups, for example when examining genotype combinations, one-way ANOVA with a trend test was used. All statistical analyses were performed using Prism 4.0 statistical software (Graphpad Inc., USA). Differences
3. Results

Allelic and genotype frequencies for various SNPs studied are shown in Table 2. The genotype distributions of GCPII (C1561T), RFC (G80A), MTR (A2756G), MTRR (A66G, C524T), MTHFR (C677T, A1298C), and TS (tandem repeats and 6 bp deletion in 3′-UTR region) in the cohort studied were in Hardy–Weinberg equilibrium.

Association of SNPs with BN-MN frequency was initially analyzed by examining effects for a single gene and single polymorphic site. For these analyses, we compared age- and gender-adjusted BN-MN cell frequency in homozygotes for the common allele with those who carried one or two copies of the rarer variant allele (Table 3). The results showed no effect of GCPII, MTRR, TS and MTHFR polymorphisms on BN-MN frequency.

A marginally significant but substantial effect of the RFC (G80A) and MTR (A2756G) polymorphisms on BN-MN frequency was observed. BN-MN frequency in individuals who carried at least one copy of the rarer G allele for MTR (A2756G) or were homozygotes for the more common G allele for RFC (G80A) had a 14% or 19% lower BN-MN frequency compared to the alternative genotypes for that SNP respectively which equated to a difference of 1.8 BN-MN and 2.0 BN-MN per 1000 BN cells respectively. It was evident from genotype combination analyses (Fig. 2) that BN-MN frequency per 1000 BN cells was highest in those with the combined GA or AG and RFC (G80A) GA or AA genotype (13.6 BN-MN per 1000 BN cells) and lowest in those with the combined MTR (2756) AA or AG and RFC (80) GG genotypes (9.5 BN-MN per 1000 BN cells) (P trend = 0.015) which equated to a difference of 4.1 BN-MN per 1000 BN cells. The effect sizes observed for the MTR (A2756G) and RFC (G80A) polymorphisms were within the range considered pathologically important which the study was designed to detect.
4. Discussion

The results of the present study suggest that only RFC A80G and MTR A2756G polymorphisms may have a significant impact on BN-MN frequency in the studied population of healthy non-smoking South Australians. RFC is required for the uptake of 5-MeTHF into cells and MTR is important as it converts 5-MeTHF to THF a form of folate, appropriate maintenance of DNA methylation as well as prevention of excessive uracil incorporation into DNA all of which may affect micronucleus formation. The RFC A80G polymorphism and in a third study in healthy subjects GG genotype was associated with increased MN frequency. There are no previous studies on the effect of the T allele in RFC A80G polymorphism and higher BN-MN frequency also comes from the observation in case-control studies showing independently that micronucleus frequency in lymphocytes and frequency of the T allele of MTR (A2756G) is increased in Alzheimer disease [40–42].

However, two other studies, one on mothers of Down syndrome children [43] and another on non-habitual drinkers [18] did not observe an association between the MTR (A2756G) polymorphism and MN frequency in lymphocytes. The lack of an association between BN-MN frequency and the GCPII (C1561T) polymorphism may have been due to reduced statistical power given the small number (N = 14) of individuals who were carriers of the T allele. Three previous studies investigated an association between the MTRR (A66G) polymorphism and MN frequency [15,16,18] with conflicting outcomes. In one study those with the AA genotype had higher MN frequency, however, this effect was only observed in current smokers, in a second study focussed on coronary artery disease patients there was no effect of the MTRR (A66G) polymorphism and in a third study in healthy subjects GG genotype was associated with increased MN frequency. There are no previous studies on the effect of MTRR C524T polymorphism and lymphocyte BN-MN frequency. Our observations support a null effect of common MTRR polymorphisms (A66G, C524T) on lymphocyte BN-MN frequency.

The lack of an effect of the MTHFR C677T polymorphism on BN-MN frequency observed in our study is consistent with (a) the lack of an effect of the T allele in in vitro studies on BN-MN frequency suggesting that genetic factors associated with longevity may also be linked with lower BN-MN frequency. Furthermore, indirect support for the association between the A allele of the MTR (A2756G) polymorphism and higher BN-MN frequency also comes from the observation in case-control studies showing independently that micronucleus frequency in lymphocytes and frequency of the A allele of MTR (A2756G) is increased in Alzheimer disease [40–42].

There are no previous reports of RFC A80G polymorphism and chromosomal instability or micronucleus formation and therefore it will be important to test whether our results can be reproduced in other populations. The association of the MTR (A2756G) AG or GG genotype with lower BN-MN frequency is consistent with the observation in a study in Bonn (Germany) which showed that carriers of the G allele for MTR (A2756G) are more likely to live to 100 years [36] given that micronucleus frequency in lymphocytes increases with age [37] and in accelerated ageing syndromes [38,39] of an effect of the T allele in other populations. The association of the A allele of MTR (A2756G) is increased in Alzheimer disease [40–42]. However, two other studies, one on mothers of Down syndrome children [43] and another on non-habitual drinkers [18] did not observe an association between the MTR (A2756G) polymorphism and MN frequency in lymphocytes.

The lack of an association between BN-MN frequency and the GCPII (C1561T) polymorphism may have been due to reduced statistical power given the small number (N = 14) of individuals who were carriers of the T allele. Three previous studies investigated an association between the MTRR (A66G) polymorphism and MN frequency [15,16,18] with conflicting outcomes. In one study those with the AA genotype had higher MN frequency, however, this effect was only observed in current smokers, in a second study focussed on coronary artery disease patients there was no effect of the MTRR (A66G) polymorphism and in a third study in healthy subjects GG genotype was associated with increased MN frequency. There are no previous studies on the effect of MTRR C524T polymorphism and lymphocyte BN-MN frequency. Our observations support a null effect of common MTRR polymorphisms (A66G, C524T) on lymphocyte BN-MN frequency.

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comparing the CC and TT genotype in which folate and riboflavin (cofactor for MTHFR) were controlled within or close to the physiological range [44] and (b) those from the only other two in vivo studies that explored the impact of the three common MTHFR polymorphisms which showed a null effect on MN frequency in healthy subjects [15–16,18]. An increase in MN frequency in coronary artery disease patients who were T allele homozygotes for the MTHFR (C677T) polymorphism has been reported but whether this observation is a consequence of disease treatment is not clear yet [15]. Ishikawa et al. reported the only other study investigating the effect of the 6bp deletion in the 3′-UTR region of TS and like us did not find an association of this deletion with expression of micronuclei in lymphocytes [18].

Although our study has the strength of considering polymorphisms across a logical sequence of genes that relate to folate uptake from the gut into blood and cells as well as metabolism of folate within the cell it also has some limitations: (a) the sample size is relatively small making it impractical to make adjustments for effects of variables other than those that are already well-established and known to affect BN-MN frequency substantially such as age and gender [37,45] and (b) the multiple assessments made might have increased the probability of detecting an association by chance. For each of the genes we investigated there was biological plausibility that common SNPs in these genes might affect folate metabolism and BN-MN induction and in this sense the impact of each SNP was investigated independently i.e. this was not a “fishing expedition” based on a global null hypothesis approach which is usually the basis for needing to perform a Bonferroni correction for multiple comparisons. A Bonferroni correction would reduce the P value if there was strong logic for this correction but the problem with this approach is that it puts the emphasis on statistical significance rather than practical (or biological/pathological) significance. The results clearly show that for the majority of SNPs studied (i.e. all excluding the GCPII C1561T and MTRA2756G SNPs) the difference for age- and gender-adjusted BN-MN between carrier and non-carrier groups was 0.9% or less (range 0.17–0.88) which is well below the biologically meaningful cut-off range of 1.8–2.9‰. This means that the practical significance of the extent of these differences is so small that designing much larger studies to achieve statistical significance in these cases is effectively futile. Nevertheless, we acknowledge the need to replicate the results in a larger cohort that would be sufficiently powered to also take into consideration possible confounding effects of other common polymorphisms such as those in DNA repair genes that are also associated with micronucleus frequency [46].

Proteins encoded by the genes we investigated and their relevant polymorphisms may influence plasma folate, vitamin B12, and homocysteine [20,47,48] depending on dietary folate and vitamin B12 intake which in turn can impact on chromosomal instability. For this reason it was important to exclude the possibility that any observed effect of folate metabolism genotypes was not attributable to differences in folate and vitamin B12 as we have in fact shown in the case of the MTR (A2756G) and RFC (G80A) genotype combination analysis (Table 4). In vitro studies similar to those performed by Kimura et al. [44] on the MTHFR C677T polymorphism and its interaction with folate and riboflavin in affecting genome damage biomarkers are also needed to verify that the MTR (A2756G) and RFC (G80A) polymorphisms do in fact influence micronucleus frequency under tightly controlled conditions and whether these effects depend on folate and/or vitamin B12 concentration. The results of our study cannot be extrapolated to individuals with different folate and vitamin B12 status because deficiency or excess of these vitamins relative to the values in our study cohort may have either amplified or dampened the impact of the MTR (A2756G) and RFC (A80G) polymorphisms on BN-MN frequency.

In conclusion, only the RFC G80A and MTR A2756G polymorphisms and their combinations appeared to produce effect sizes in BN-MN frequency that were of pathological significance as well as statistical significance, given the limited power of the study. These results are important because they inform us on which SNPs should be given priority in future larger studies in South Australia.

Conflict of interest

None declared.

Acknowledgements

We are grateful to all the volunteers who donated their blood for this study and acknowledge the important contribution of Julie Turner and Carolyn Salisbury for performing the CBMN assays.

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