Impact of folate and homocysteine metabolism on human reproductive health

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Folates belong to the vitamin B group and are involved in a large number of biochemical processes, particularly in the metabolism of homocysteine. Dietary or genetically determined folate deficiency leads to mild hyperhomocysteinemia, which has been associated with various pathologies. Molecular mechanisms of homocysteine-induced cellular dysfunction include increased inflammatory cytokine expression, altered nitric oxide bioavailability, induction of oxidative stress, activation of apoptosis and defective methylation. Whereas the involvement of folate metabolism and homocysteine in ageing-related diseases, in several developmental abnormalities and in pregnancy complications has given rise to a large amount of scientific work, the role of these biochemical factors in the earlier stages of mammalian reproduction and the possible preventive effects of folate supplementation on fertility have, until recently, been much less investigated. In the present article, the possible roles of folates and homocysteine in male and female subfertility and related diseases are systematically reviewed, with regard to the epidemiological, pathological, pharmacological and experimental data of the literature from the last 25 years.

Key words: fertility/folates/homocysteine/MTHFR polymorphism/human reproduction

Introduction

Folates are a group of inter-convertible co-enzymes, differing by their oxidation state, number of glutamic acid moieties and one-carbon substitutions. They are involved in amino acid metabolism, purine and pyrimidine synthesis and methylation of a large number of nucleic acids, proteins and lipids. Of particular interest is the interface between folate metabolism and the homocysteine/methionine cycle. Homocysteine, a sulfhydryl-containing amino acid that is not used in protein synthesis, originates exclusively from the one-carbon-donating metabolism of methionine, and it is remethylated into methionine with folates acting as methyl donors (Lucock, 2000).

Over the past decade, there has been a growing body of evidence that even a moderately elevated serum homocysteine concentration is associated with an increased risk of ageing-related diseases, such as atherosclerotic, thromboembolic and neurodegenerative disorders, and also with early pathological events of life (Herrmann, 2001; Gueant et al., 2003). The latter category includes a number of developmental abnormalities, particularly neural tube defects, as well as late pregnancy complications, such as pre-eclampsia, abruptio placenta, intrauterine growth retardation, preterm birth and intrauterine fetal death (Eskes, 2000; Nelen, 2001; Hague, 2003; Steegers-Theunissen et al., 2004; Tamura and Picciano, 2006).

Whereas a large amount of scientific work has investigated the roles of folate metabolism and hyperhomocysteinemia in malformations and pathologies of the ongoing pregnancy, there is only little information on a possible involvement of these biochemical phenomena in the earlier stages of reproductive physiology and in related diseases. In the present article, current data on the influence of folate metabolism on male and female reproductive tracts and fertility are reviewed by means of a systematic review of the Medline database-indexed literature since 1980.

Biochemical background

In most mammalian cells, accumulating homocysteine is removed either by remethylation into methionine or by trans-sulfuration into cysteine (Scott and Weir, 1998; Fowler, 2005). In the trans-sulfuration pathway (Figure 1), homocysteine is condensed with serine in an irreversible reaction catalyzed by cystathionine-beta-synthase (CBS) to form cystathionine, which in turn is reduced to 225
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Folate metabolism and reproduction


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Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects

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Gene–environment interactions play an important role in folate metabolism, with a potential impact on human health. Deficiencies in the uptake of key micronutrients and variant genotypes can affect the folic acid cycle, modulating methyl group transfer in key processes and leading to increased cancer risk and Down syndrome incidence. So far, the significance of folate status and metabolic genotypes on baseline levels of DNA damage in normal individuals has not been fully elucidated. In this study, the possible modulation of SCE, micronuclei and tail moment values in peripheral lymphocytes by plasma levels of folic acid, homocysteine and vitamin B12, and by the methylene-tetrahydrofolate reductase (MTHFR) C677T and methionine synthase reductase (MTRR) A66G polymorphisms was investigated in 191 healthy subjects. The results obtained show a highly significant ($P = 0.001$) positive association between plasma levels of vitamin B12 and frequencies of both SCE and high frequency cells (HFC, above 90 percentile) in smokers. No significant effect was observed in non-smokers. Moreover, after correction for age, gender and GSTM1 genotype, a significant association ($P = 0.026$) between the MTRR 66GG variant genotype and higher micronucleus rates was observed. Tail moment values were not affected by any of the independent variables considered. Overall, the results obtained suggest that both folate status and relevant metabolic genotype can influence background levels of DNA damage in normal subjects. The significant association observed in smokers between plasma vitamin B12 and SCE frequencies may highlight the effect of methylation status on DNA damage and repair, although the role of other, unidentified dietary factors cannot be ruled out. At the same time, micronucleus data indicate that the MTRR 66GG variant may represent another individual trait of relative genomic instability, thus supporting epidemiological data on increased risk of Down syndrome conception in MTRR 66GG subjects.

Introduction

Among the factors associated with increased risk of cancer, diet accounts for an attributable risk estimated at ~20–40% (1). The impact of diet on human health is due both to the exposure to carcinogenic contaminants and to the abnormal uptake of key micronutrients involved in DNA maintenance (2). In this respect, the metabolic pathway of folate, which is impaired by an unbalanced diet, is thought to influence DNA stability in two different ways (3,4) (and references therein). The first is related to the role of folate in one carbon unit transfer during de novo synthesis of nucleotides. Low levels of 5,10-methylenetetrahydrofolate (5,10-methylene-THF), the cofactor of thymidylate synthase, depress thymidylate synthesis, leading to an increased dUMP/dTMP ratio and increased dUTP misincorporation in DNA. The removal of dUTP by DNA-glycosylase may lead to single and double strand breaks; furthermore, the unbalanced nucleotide pool resulting from inefficient thymidylate synthase activity can increase DNA misrepair, contributing to the overall level of DNA damage in the cell. The second way in which folate metabolism may affect DNA maintenance involves the production of S-adenosyl methionine (SAM), the methyl donor of most methylation processes, including CpG methylation. 5,10-Methylene-THF, after conversion into 5-methyltetrahydrofolate (5-methyl-THF) by methylenetetrahydrofolate reductase (MTHFR), provides the methyl group for methylation of homocysteine to methionine, the precursor of SAM, by methionine synthase (MTR). Thus, low intracellular 5,10-methylene-THF is associated with low SAM production; this may lead to DNA undermethylation, a cause of abnormal gene expression (5) and chromosome segregation (6).

Evidence supports the key role of folate status in human health. An unbalanced diet with reduced uptake of folic acid was associated with increased risk of colon cancer (7). In studies on human donors, low levels of serum folic acid were associated with a higher misincorporation of uracil into DNA as well as with increased incidence of micronuclei in peripheral lymphocytes (8). Micronuclei were also positively correlated to serum homocysteine levels, a marker of folate deficiency (9,10). The role of folic acid in DNA integrity was also demonstrated in in vitro studies, where low folic acid concentrations in the medium produced higher uracil misincorporation, micronuclei and DNA strand breaks as detected by comet assay (11,12).

In addition to dietary uptake of micronutrients such as folic acid and vitamin B12 (the coenzyme of MTR), also some genetic polymorphisms may affect folic acid metabolism, modulating its effect on human health. Both the polymorphisms of MTHFR C677T and the methionine synthase reductase (MTRR) A66G (13) were associated, in some studies, with increased maternal risk of having a child with Down syndrome (14,15), as well as with increased risk of neonatal spina bifida (16). On the other hand, lower colon cancer risk was associated with the MTHFR 677TT genotype (17,18), and reduced risk of

Abbreviations: MEIA, microparticle enzyme immunoassay; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; NDI, nuclear division index; PRI, proliferation index; RCF, red blood cell folate; RIA, radioimmunoassay; SAM, S-adenosyl methionine; THF, tetrahydrofolate.
Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects

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into the cell (44). Perturbation of this pathway may reduce global methylation of repeated sequences, including those localized in the centromeric region, and disturbing the apparatus for chromosome segregation (45,46). On the other hand, factors favouring DNA methylation could modulate gene expression or, as suggested by the results of this study, exert an indirect influence on DNA integrity, mediated by the interaction with exogenous factors (47).

Several case-control studies indicate that the MTHFR C677T polymorphism can modify cancer risk (48), and that MTHFR 677TT subjects with low serum folic acid show DNA hypomethylation (49,50). However, no modulation of background genetic damage by the MTHFR polymorphism was observed in this study. Indeed, recent in vitro studies on folic acid deficiency and DNA damage in lymphocytes failed to discriminate subjects with different MTHFR genotype (12,23). These results seem to indicate a marginal influence of the MTHFR genotype on DNA integrity, even though it is possible that unphysiological levels of methionine and riboflavin in culture medium may have biased the results of the in vitro studies.

Previous investigations on the Australian population indicated that micronucleus frequency in men was also significantly increased with increasing plasma homocysteine (9,10). In this study, no significant correlation between micronuclei and homocysteine was observed, once that the effect of other correlated variables (age, gender) was taken into account. However, a direct effect of homocysteine on micronucleus formation in vitro could not be demonstrated (51), and it is possible that in different populations (e.g. Australian versus Italian), plasma homocysteine may be associated with different confounding factors, e.g. related to different dietary habits.

Finally, even though it was recently estimated that the recommended daily intake of micronutrients may be suboptimal for the purpose of protection from genotoxic damage (4), the results of this study indicate that the relationship between genetic integrity and folate status may be complex, modulated by exogenous factors such as tobacco smoke. Yet, both the effect of vitamin B12 on SCE, and that of the MTRR genotype on micronuclei, confirm that folate metabolism plays a critical role in the maintenance of genomic integrity. Further investigation on the relationships between DNA methylation/damage and folate status in different genetic backgrounds are necessary to fully elucidate the consequences of unbalanced folate metabolism.

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References

Meta-Analysis


Impact of Violations and Deviations in Hardy-Weinberg Equilibrium on Postulated Gene-Disease Associations

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The authors evaluated whether statistically significant violations of Hardy-Weinberg equilibrium (HWE) or the magnitude of deviations from HWE may contribute to the problem of replicating postulated gene-disease associations across different studies. Forty-two gene-disease associations assessed in meta-analyses of 591 studies were examined. Studies with disease-free controls in which HWE was violated gave significantly different results from HWE-conforming studies in five instances. Exclusion of the former studies resulted in loss of statistical significance of the overall meta-analysis in three instances and more than a 10% change in the summary odds ratio in six. Exclusion of HWE-violating studies changed the formal significance of the estimated between-study heterogeneity in three instances. After adjustment for the magnitude of the deviation from HWE for the controls, formal significance was lost in another three instances. Studies adjusted for the magnitude of deviation from HWE tended to become more heterogeneous among themselves, and, for seven gene-disease associations, between-study heterogeneity became significant, while it was not so in the unadjusted analyses. Gene-disease association studies and meta-analyses thereof should routinely scrutinize the potential impact of HWE violations as well as nonsignificant deviations from the exact frequencies expected under HWE. Postulated genetic associations with modest-sized odds ratios and borderline statistical significance may not be robust in such sensitivity analyses.

Abbreviations: HWE, Hardy-Weinberg equilibrium; ID, (operational) identification number.

Genetic associations for complex diseases are important to establish because they are expected to cumulatively account for a substantial proportion of susceptibility to many diseases of considerable public health impact (1, 2). However, concerns are increasing about the lack of replication of proposed gene-disease associations (3–5). Various reasons have been proposed for this phenomenon, including, among others, exaggerated early results (3), publication bias and time-lag bias against “negative” studies (3, 6), differences between small and larger studies (7), and population stratification and “racial/ethnic” heterogeneity (8). Empirical evidence has been accumulating on the relative role of some of these potential problems (3, 4, 7–9).

An additional major theoretical concern is that associations may be spurious if the distribution of genotypes in the healthy control groups in genetic case-control studies
HWE may be violated because of genotyping error, chance, inbreeding, nonrandom mating, differential survival of marker carriers, genetic drifting, population stratification, or combinations of these reasons. Some of these effects may also occasionally act in different directions, canceling each other. Unfortunately, most gene-disease association studies report very limited information to provide any detailed insight into these potential problems. Thus, also at a meta-analysis level, typically there is no way to decipher for which of the above reasons the violation has occurred, except perhaps for population stratification under special conditions (57). For this reason, sensitivity analyses including and excluding the HWE-deviating studies have been recommended (58). However, most published meta-analyses systematically neglect such analyses (59). Adjustments for the magnitude of deviation from HWE are rarely performed in primary studies, and, to date, these corrections have not been introduced at all into meta-analyses, to our knowledge. Such adjustments are as useful to perform as exclusion of HWE-violating studies. The two corrective measures may offer complementary information.

Whenever HWE-related corrective measures resulted in loss of the significance of an association, the change in the estimates of the odds ratio was relatively small with these corrections. However, most effect sizes in genetic epidemiology are very modest anyway (3, 7–9). Formal statistical significance is routinely considered important for deciding whether or not the probed association is present. Our finding suggests that gene-disease associations should be pronounced with extra caution when \( p \) values are not much smaller than 0.05. Relatively minor deviations in the genotype frequencies could change whether or not formal

**FIGURE 3.** Meta-analyses before and after adjustments for departures from the Hardy-Weinberg law. Each meta-analysis is preceded by the (operational) identification number (ID) given in table 1, the first author of the study and the year of publication (e.g., ’98 = 1998), and the reference number. The lower set of 11 meta-analyses used allele-based contrasts. For each meta-analysis, summary odds ratios and their 95% confidence intervals (CIs) are depicted. * meta-analyses in which formal statistical significance was lost after adjustment for deviations from Hardy-Weinberg equilibrium (HWE).


52. Wheeler JG, Keavney BD, Watkins H, et al. Four paraoxonase combinations of genotypes: a biallelic locus (alleles A and a) with a disease (by genotype groups, we refer to genotypes: AA, Aa, or aa; or combinations of genotypes: AA + Aa, Aa + aa, etc.). The Lathrop correction for deviations from HWE in the controls calculates the adjusted odds ratio (ORadj) by using the HWE-predicted genotype counts in the control group (controlsHWEpredicted i) instead of the observed ones: ORadj = (casesObserved × controlsG1 HWEpredicted) / (casesG1 Observed × controlsG2 HWEpredicted).


APPENDIX

To correct for departures from HWE for the controls for genotype-based contrasts (22), let G1 and G2 be the genotype groups that are contrasted in an association study of a biallelic locus (alleles A and a) with a disease (by genotype groups, we refer to genotypes: AA, Aa, or aa; or combinations of genotypes: AA + Aa, Aa + aa, etc.). The Lathrop correction for deviations from HWE in the controls calculates the adjusted odds ratio (ORadj) by using the HWE-predicted genotype counts in the control group (controlsHWEpredicted i) instead of the observed ones: ORadj = (casesObserved × controlsG1 HWEpredicted) / (casesG1 Observed × controlsG2 HWEpredicted). The variance of the natural logarithm of the adjusted odds ratio would then become Var = 1/casesG1 Observed + 1/casesG2 Observed + vG1 vs G2, where vG1 vs G2 is an estimate of the variance of the HWE-predicted control counts depending on the specific contrast G1 vs G2. More specifically, if pA, pB are the corresponding allele frequencies in the controls, and NA and Np are the total number of alleles in the controls, then vG1 vs G2 is given by the following formula for the different types of contrasts:

\[
\begin{align*}
v_{AA, VS, AA + AA} &= 4\left(1 - p_A\right)\left(1 + p_A\right)^2 N_A \\
v_{AA, VS, AA + Aa} &= 4\left(1 - p_A\right)\left(1 + p_A\right)^2 N_a \\
v_{AA, VS, AA + aa} &= \left(1 - 2p_A\right)/N_A + \left(1 - 2p_A\right)/N_a/2p_A p_A \\
v_{AA, VS, AA + aa} &= 4/N_A + 4/N_a
\end{align*}
\]
The M235T Polymorphism in the AGT Gene and CHD Risk: Evidence of a Hardy-Weinberg Equilibrium Violation and Publication Bias in a Meta-Analysis

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Abstract

Background: The M235T polymorphism in the AGT gene has been related to an increased risk of hypertension. This finding may also suggest an increased risk of coronary heart disease (CHD).

Methodology/Principal Findings: A case-cohort study was conducted in 1,732 unrelated middle-age women (210 CHD cases and 1,522 controls) from a prospective cohort of 15,236 initially healthy Dutch women. We applied a Cox proportional hazards model to study the association of the polymorphism with acute myocardial infarction (AMI) (n = 71) and CHD. In the case-cohort study, no increased risk for CHD was found under the additive genetic model (hazard ratio [HR] = 1.20; 95% confidence interval [CI], 0.86 to 1.68; P = 0.28). This result was not changed by adjustment (HR = 1.17; 95% CI, 0.83 to 1.64; P = 0.38) nor by using dominant, recessive and pairwise genetic models. Analyses for AMI risk under the additive genetic model also did not show any statistically significant association (crude HR = 1.14; 95% CI, 0.93 to 1.39; P = 0.20). To evaluate the association, a comprehensive systematic review and meta-analysis were undertaken of all studies published up to February 2007 (searched through PubMed/MEDLINE, Web of Science and EMBASE). The meta-analysis (38 studies with 13284 cases and 18722 controls) showed a per-allele odds ratio (OR) of 1.08 (95% CI, 1.01 to 1.15; P = 0.02). Moderate to large levels of heterogeneity were identified between studies. Hardy-Weinberg equilibrium (HWE) violation and the mean age of cases were statistically significant sources of the observed variation. In a stratum of non-HWE violation studies, there was no effect. An asymmetric funnel plot, the Egger’s test (P = 0.066), and the Begg-Mazumdar test (P = 0.074) were all suggestive of the presence of publication bias.

Conclusions/Significance: The pooled OR of the present meta-analysis, including our own data, presented evidence that there is an increase in the risk of CHD conferred by the M235T variant of the AGT gene. However, the relevance of this weakly positive overall association remains uncertain because it may be due to various residual biases, including HWE-violation and publication biases.

Introduction

Angiotensinogen (AGT) is a liver protein that interacts with renin to produce angiotensin I, the pro-hormone of angiotensin II. Angiotensin II is the major effector molecule of the renin-angiotensin-aldosterone system (RAAS) and plays a key role in the regulation of blood pressure (BP) by increasing vascular tone and promoting sodium retention. Genetic variants in the angiotensinogen gene modify the plasma concentration of angiotensinogen, which has been directly related to arterial blood pressure [1]. The molecular variant (M235T) of the AGT gene, encoding a threonine instead of a methionine at residue 235 of the mature protein, has been associated with a higher plasma AGT level and higher BP in patients homozygous for the T allele and occurs among various ethnic populations [1–3]. In a meta-analysis, the TT genotype was associated with a 32% increase in the risk of hypertension in white people but not in non-white people, when compared with the MM genotype [4].

Given the importance of hypertension in the occurrence of coronary heart disease [5], this finding suggests that this polymorphism may be related to increased risk of CHD. A few studies [6–8], including recent publications, [9,10] have found that there is an association of the M235T AGT variant with increased CHD risk; however, this relationship was not confirmed in several other studies [11–13] as well as in a meta-analysis [14]. Marked ethnic differences in the frequency of the T allele, small sample
Moreover, there was evidence for publication bias in the meta-analysis. Taken together, these findings point to a violation of HWE and publication biases as the potential explanations for the results observed in the meta-analysis.

Some aspects of the current meta-analysis need to be considered to appreciate the findings. First, it might not be very practical to adjust for violation of HWE in the studies that mentioned that the violation is not due to genotyping errors. However, in the current meta-analysis, the HWE-violated studies that were included in the pooled estimate did not provide any reason for the violation. Therefore, we performed sensitivity analyses by using HWE-adjusted ORs and corresponding variances. Thereafter, a smaller overall effect was seen under most of the genetic models. Second, the power of tests for HWE and the power to detect genotyping errors are low. Therefore, the inability to detect a deviation from the HWE does not mean that there is no deviation, nor does it rule out the presence of genotyping errors, especially for small sample sizes. Third, our meta-analysis was based on published studies and we did not have access to the original data. However, it could be possible that an association between the genotype and disease exists in certain contexts rather than in all people studied. For example, a case-control study showed that the TT genotype was associated with an increased risk of CHD and MI only in smokers [33]. Finally, in all meta-analyses of gene-disease association studies, the inclusion criteria of cases and controls can be a potentially confounding factor. In this meta-analysis, cases were well defined and the source of controls was not a significant source of variation. However, the advantages of this study were the large sample size of the meta-analysis of 38 studies with 13284 cases and 18722 controls, which was twice the number of studies and sample sizes that had been reported in the previous meta-analysis [14], the exploration of potential sources of heterogeneity in the meta-analysis, and the evaluation of the association under different modes of inheritance.

Approximately 10% of gene-disease association studies are affected by statistically significant deviation from HWE, which could result from genotyping errors, chance, inbreeding, non-random mating, differential survival of marker carriers, genetic drift, population stratification, or a combination of these reasons [20,60]. Of these, genotyping error could be avoided by using standard genotyping methods and performing quality assessment. It has been recommended that authors specify the quality measures for the genotyping analysis, such as the blinding of laboratory staff to the donor subjects and hypotheses being investigated, procedures for establishing duplicates, degree of reproducibility between quality control replicates, and the inspection for conformity to HWE [61]. In the current meta-analysis, in studies where the blinding of genotyping staff was not reported, a statistically significant increased risk of CHD was found, while those that used blinding methods did not find a significant association. Moreover, for studies without regenotyping of a random sub-sample, a significant increase in CHD risk was found, but not for studies that performed regenotyping. Although overlapping confidence intervals for before-mentioned risks indicate caution in any interpretations, no report on blinding and regenotyping can point towards an uncertainty in quality control of genotyping in these studies. However, violation of HWE, which tends to inflate the chance of a false positive association, may be the strongest indicator of genotyping error [62].

Violation of HWE cannot solely explain the observed between-study variation in gene-disease association studies. The large between-study heterogeneity presented in most meta-analyses could be due to true heterogeneity (i.e., racial differences or differences in gene-environment interactions among various populations) or bias [63]. Bias, which could invalidate the results of the studies, should, therefore, be explored in detail. Biological plausibility, publication bias, selection bias, biased definition of cases, biased selection of controls, and population stratification should be assessed [63]. In this meta-analysis, we found strong evidence for publication bias. This is said to occur when the chance of the publication of a smaller study increases when it shows a stronger effect. Further exploration for sources of biases among studies showed that the selection of controls was not biased. However, using different case definitions resulted in a significant difference in the risk of CHD between those studies using WHO criteria and those using clinically diagnoses of CHD. Studies using definition of cases based on coronary angiography or based on WHO criteria had the same results. Considering a multivariate model in the meta-regression results, case definition was not a significant source of bias in the meta-analysis, while the different...
ABSTRACT

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Material and methods: We analyzed the distribution of this single nucleotide polymorphism in the MTHFR gene in a case group of 66 infertile Romanian patients with idiopathic azoospermia or severe oligozoospermia and a control group of 67 Romanian men to explore the possible association of the A1298C polymorphism and male infertility. Using the polymerase chain reaction—restriction fragment length polymorphism technique (PCR-RFLP), the allele and genotype distribution of SNP A1298C in the MTHFR gene were investigated in both patients and controls.

Outcomes: The frequencies of the polymorphism in infertile patients were not significantly higher than those in controls.

Conclusions: Our findings suggest that there is no significant association of SNP A1298C in the MTHFR gene with azoospermia or oligozoospermia, indicating that this polymorphism would not be a genetic risk factor for male infertility in our study group. It may be necessary to enlarge the study groups in order to obtain more significant conclusions and to evaluate other polymorphisms in genes that code for key enzymes in the folate and homocysteine metabolism, for being able to interpret the eventual complex gene-gene interactions with possible implications in the studied pathology.

Key words: homocysteine, folate, azoospermia, DNA methylation

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Methylenetetrahydrofolate reductase A1298C polymorphism and male infertility in a Romanian population group

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**INTRODUCTION**

Couple infertility is a global health problem and according to the World Health Organization approximately one couple in seven is affected by fertility or subfertility problems (1). Male infertility in humans has been acknowledged as the cause of couple’s inability to have children in 20-50% of total cases (2).

The most common non-genetic causes of male infertility are: hypogonadism, testicular maldescence, structural abnormalities of the male genital tract, genital infections, previous scrotal or inguinal surgery, varicoceles, chronic illness, medication and exposure to chemicals. However in about 40% of cases no cause was found related to infertility, hence launching the idea that a high number of idiopathic male infertility cases could be attributed to genetic factors. Genetic abnormalities were identified in men with unexplained oligozoospermia and azoospermia, including numerical and structural chromosomal abnormalities (3,4), deletions of the azoospermia factor region (AZF) of the Y chromosome or translocations between the Y chromosome and other chromosomes (5-7), mutations in the cystic fibrosis conductance regulator (CFTR) gene, commonly associated with congenital vas deferens abnormalities (8,9) and also other genetic factors (10). It was observed that some abnormalities associated with infertility are inherited, like reciprocal and Robertsonian translocations and CFTR mutations (11), while the majority of numerical chromosome abnormalities and AZF deletions are de novo events in the parental germ cells.

Folates are a group of inter-convertible coenzymes, differing by their oxidation state, number of glutamic acid moieties and one carbon substitutions. They are involved in amino acid metabolism, purine and pyrimidine synthesis and methylation of a large number of proteins, lipids, and nucleic acids as well. The relation between folate metabolism and the methionine/homocysteine pathway is particularly important. Homocysteine, a sulfhydryl-containing amino acid that is not used in protein synthesis, originates exclusively from the one-carbon metabolism of methionine, and it is remethylated into methionine with folates acting as methyl donors (12). In the last decade increased plasmatic levels of homocysteine have been found to be associated with an increased risk for several diseases, such as atherosclerotic, thromboembolic and neurodegenerative disorders, and also with early pathological events of life (13,14). The latter category of disorders includes the following: neural tube defects, late pregnancy complications such as pre-eclampsia, abruptio placenta, intrauterine growth retardation, preterm birth and intrauterine fetal death (15-19). However, although the recent progress in understanding the physiopathology of hyperhomocysteinemia – induced health events, there is only little information on the role of folates/homocysteine on male reproduction.

Within the folate metabolic cycle the MTHFR (methyleneetetrahydrofolate reductase) gene encodes a key regulatory enzyme responsible for the reduction of 5, 10-methyltetrahyrofolate, thus catalyzing the only reaction in the cell that ultimately generates 5-methyltetrahydrofolate, the biologically active folate derivative. The importance of folate metabolism is related to its function in providing one-carbon units for nucleic acids bases synthesis as well as for the synthesis of S-adenosylmethionine, the universal methyl donor for several biological methylation reactions.

Within the MTHFR gene several SNPs (single nucleotide polymorphisms) have been described. Martin et al. (20) resequenced the MTHFR gene product, and found a total number of 65 polymorphisms, 11 of which were non-synonymous cSNPs. A transition from cytosine to thymine at the 677 position of the MTHFR gene causes enzyme thermolability and reduced activity (21), therefore, impairments of MTHFR function, such as those associated with the presence of the C677T polymorphic site, are critical for altering nucleic acid metabolic pathways. Van der Put et al. (22) identified another polymorphism of the MTHFR gene, a 1298A-C mutation resulting in a glut-ala substitution at position 429. It must be taken into account that whereas the C677T transition occurs within the predicted catalytic domain of the MTHFR enzyme, the A1298C transition is located in the presumed regulatory domain. The A1298C mutation resulted in decreased enzyme activity, which was more pronounced in the homozygous than heterozygous state.

The biochemical properties of the products of the C677T and A1298C polymorphisms...
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METHYLENETETRAHYDROFOLATE REDUCTASE A1 298C POLYMORPHISM AND MALE INFERTILITY IN A ROMANIAN POPULATION GROUP

were studied by Yamada et al. (23), who observed that the A222V MTHFR enzyme corresponding to the C677T transition had an enhanced propensity to dissociate into monomers and lose its FAD (flavin adenin dinucleotide) cofactor on dilution, while the E429A protein had biochemical properties indistinguishable from the wildtype enzyme.

MATERIALS AND METHODS

Our study was performed on a group of 66 infertile Romanian patients from which 54 were diagnosed with idiopathic azoospermia and 12 with severe oligozoospermia, and a control group of 67 Romanian men with at least 1 child. Patients with a history of varicocele, congenital abnormalities, urogenital infections, and undescended testicles were excluded from the test after examination by a specialist. Also after performing chromosomal and molecular analyses, patients with chromosomal abnormalities, microdeletions in the AZF region of the Y chromosome were excluded from the study group. Informed consent regarding genetic testing was obtained from all study subjects. For genetic testing, 3ml of peripheral blood was extracted in EDTA to prevent blood clotting. Genomic DNA was extracted from blood leukocytes contained in a volume of 300µl using a commercially available extraction kit (Wizard Genomic DNA Purification Kit, Promega®). The presence of the MTHFR A1298C polymorphism.

was detected by means of molecular genetic techniques, respectively polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) by modifying a previously described protocol, van der Put et al. (22). The PCR amplification reaction was performed in a total volume of 25µl containing approximately 100ng of genomic DNA, 12.5µl PCR Master Mix (Fermentas MBI, Lituania®), 1µl BSA (Bovine Serum Albumine, Fermentas MBI, Lituania®) solution 2 mg/ml, 8 µM of each primer, forward and reverse (Eurogentec, Belgium®) and water free of nucleases to complete the 25µl volume. The PCR reactions were performed in a gradient thermocycler (Mastercycler Gradient, Eppendorf®) by using the following primer pairs: 5’-CTTTGGGGAGCTGAAGGACTACTAC-3’ and 5’-CACTTTGTGCACCATCTCGGTGGT-3’ under the following conditions were: an initial step consisting in denaturation for 5 min at 95°C, extension for 2 min at 55°C, extension for 2 min at 72°C, followed by 35 cycles of denaturation at 95°C for 75 s, annealing at 55°C for 75 s, extension at 72°C for 90 s, and a final extension time of 6 min at 72°C. The amplified fragment of 163 bp was digested with MboII endonuclease (Fermentas MBI, Lituania®). The A1298C mutation abolishes an MboII restriction site. Digestion of the 163-bp fragment of the 1298 AA genotype gives five fragments, of 56, 31, 30, 28 and 18 bp, whereas the 1298CC genotype results in four fragments, of 84, 31, 30 and 18 bp. The digested fragments were resolved in a 3% MetaPhor gel (Lonza®, Basel, Switzerland), stained with ethidium bromide and then visualized on a UV transilluminator Vilber Lourmat Imaging System®, Marne-la-Vallée, France. The observed alleles and genotypes frequencies were calculated for both groups and the Chi-square test for deviation was performed in order to establish if the genotype distribution in the studied population were in Hardy-Weinberg equilibrium. A comparison of the results between the study group and control group was made and the differences were tested for significance using the Chi-Square test of the statistical software SPSS Statistics 17.0.

OUTCOMES

The genotype and allelic frequencies obtained for the A1298C SNP are presented in table I. We performed association tests between the normal homozygote status (AA genotype) with the heterozygous status (AC genotype), mutant homozygous status (CC genotype) and carrier of at least one mutant allele (heterozygous AC and homozygous CC genotypes), respectively. The observed genotypes frequencies among the study groups were in agreement with Hardy-Weinberg equilibrium ($\chi^2=2.756$, $p=0.0969$) while the genotypes frequencies of the MTHFR A1298C polymorphism were 3% and 43.9% for the CC and AC genotype respectively, among the idiopathic azoospermia and severe oligozoospermia (AZF) group and 3% and 38.8%, respectively among the control group.

After applying the statistical Chi-square test to the observed genotypes all the $p$ values obtained were >0.05, considered not statistically significant. Also, the odds-ratios for all the association tests have been around the value of 1.2, revealing that the A1298C SNP in the MTHFR gene is distributed similarly in the two study groups. In our study we obtained a similar allelic frequency (0.24) of the A1298C SNP found in previous published articles.

<table>
<thead>
<tr>
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<th>AZF group n (%)</th>
<th>Control group n (%)</th>
<th>OR (95%CI)</th>
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<tbody>
<tr>
<td>Total no. of subjects</td>
<td>66 (100)</td>
<td>67 (100)</td>
<td>1.243 (0.6178-2.50)</td>
<td>0.542</td>
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was detected by means of molecular genetic techniques, respectively polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) by modifying a previously described protocol, van der Put et al. (22). The PCR amplification reaction was performed in a total volume of 25µl containing approximately 100ng of genomic DNA, 12.5µl PCR Master Mix (Fermentas MBI, Lituanian), 1µl BSA (Bovine Serum Albumine, Fermentas MBI, Lituania®) solution 2 mg/ml, 8 µM of each primer, forward and reverse (Eurogentec, Belgium®) and water free of nucleases to complete the 25µl volume. The PCR reactions were performed in a gradient thermocycler (MastercyclerGradient, Eppendorf®) by using the following primer pairs: 5′-CTTTGAGGACGCTGA AGGACTACTAC-3′ and 5′-CAGCTTGTGAC-CATCCGGTGG-3′ under the following conditions were: an initial step consisting in desaturation for 5 min at 95°C, annealing for 2 min at 55°C, extension for 2 min at 72°C, followed by 35 cycles of desaturation at 95°C for 75 s, annealing at 55°C for 75 s, extension at 72°C for 90 s, and a final extension time of 6 min at 72°C. The amplified fragment of 163 bp was digested with MboII endonuclease (Fermentas MBI, Lituanian). The A1298C mutation abolishes an MboII restriction site. Digestion of the 163-bp fragment of the 1298 AA genotype gives five fragments, of 56, 31, 30, 28 and 18 bp, whereas the 1298CC genotype results in four fragments, of 84, 31, 30 and 18 bp. The digested fragments were resolved in a 3% MetaPhor gel (Lonza®, Basel, Switzerland), stained with ethidium bromide and then visualized on a UV transilluminator VilbertLourmat Imaging System®, Marne-la-Vallée, France. The observed alleles and genotypes frequencies were calculated for both groups and the Chi-square test for deviation was performed in order to establish if the genotype distribution in the studied population were in Hardy-Weinberg equilibrium. A comparison of the results between the study group and control group was made and the differences were tested for significance using the Chi-Square test of the statistical software SPSS Statistics 17.0.

**OUTCOMES**

The genotype and allelic frequencies obtained for the A1298C SNP are presented in table 1. We performed association tests between the normal homozygote status (AA genotype) with the heterozygous status (AC genotype), mutant homozygous status (CC genotype) and carrier of at least one mutant allele (heterozygous AC and homozygous CC genotypes), respectively. The observed genotypes frequencies among the study groups were in agreement with Hardy-Weinberg equilibrium (χ²=2.756, p=0.0969) while the genotypes frequencies of the MTHFR A1298C polymorphism were 3% and 43.9% for the CC and AC genotype respectively, among the idiopathic azoospermia and severe oligozoospermia (AZF) group and 3% and 38.8%, respectively among the control group. After applying the statistical Chi-square test to the observed genotypes all the p values obtained were >0.05, considered not statistically significant. Also, the odds-ratios for all the association tests have been around the value of 1.2, revealing that the A1298C SNP in the MTHFR gene is distributed similarly in the two study groups. In our study we obtained a similar allelic frequency (0.24) of the A1298C SNP found in previous published articles.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>AZF group n (%)</th>
<th>Control group n (%)</th>
<th>OR (95%CI)</th>
<th>p value</th>
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<tr>
<td>Total no. of subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>66 (100)</td>
<td>67 (100)</td>
<td>1.243 (0.6178-2.50)</td>
<td>0.542</td>
</tr>
<tr>
<td>AC</td>
<td>35 (53)</td>
<td>39 (58.2)</td>
<td>1.114 (0.1489-8.34)</td>
<td>0.916</td>
</tr>
<tr>
<td>CC</td>
<td>29 (43.9)</td>
<td>26 (38.8)</td>
<td>1.234 (0.6217-2.448)</td>
<td>0.548</td>
</tr>
<tr>
<td>AC+CC</td>
<td>32 (46.9)</td>
<td>28 (41.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1. MTHFR A1298C genotype and allele frequencies**
In the present study, we evaluated for the first time the possibility of an association between the A1298C SNP in the MTHFR gene and male infertility in a Romanian population group. A few previous studies have evaluated the association of MTHFR C677T polymorphism in infertile patients from Germany, Netherlands, Italy, India, South Korea and China (24-30). Five of them (24, 27-29) have reported an association between these polymorphisms in the MTHFR gene and male infertility. However, the MTHFR A1298C SNP has been studied less. Varinderpal et al. (2) reported no association between the A1298C SNP and male infertility in an Indian study group; while another study done on Chinese infertile men also (29) concluded that there is no association between this SNP and the idiopathic cases of male infertility.

Considering the fact that folate deficiency has been shown to reduce the proliferation of various cell types (31) and also that it is already established that folate intake is very important for male infertility, future studies need to focus on the relation between idiopathic cases of infertility, genetic risk factors and the nutritional status of subjects; dietary habits which are particular in the country where the study is conducted influence plasma levels of homocysteine and folates.

It has already been shown that sperm concentration is increased by folic acid and zinc sulphate treatment (25). Also in the case of altered folate status due to reduced MTHFR enzyme activity, epigenetic alterations in DNA must be taken into account as important etiological factors. DNA methylation typically occurs in CpG dinucleotide rich regions, CpG islands, highly conserved sequences in promoter regions or first exons of genes (32). Because of the strong correlation between DNA methylation in promoter regions and transcriptional repression (32), DNA methylation appears to be a fundamental as well as potentially reversible mechanism for epigenetic control of gene expression. There is accumulating evidence that hypermethylation is involved in carcinogenesis since this phenomenon contribute to suppression of gene transcription (33).

Compared to other types of pathologies, vascular, neurodegenerative were wide genome association studies are being used to determine possible risk factors, until this date there is only one study of this type published by Aston et al. (34) on male infertility which investigated 370,000 SNPs and found 20 SNPs significantly associated with idiopathic forms of male infertility. However this pilot study emphasizes the fact that without proper financial support genome wide association studies are not feasible and that the candidate gene approach is still required if we are to uncover the molecular mechanisms of male infertility.

FIGURE 1. DNA electrophoresis of MTHFR A1298C on 3% MetaPhor gel: M 50 pb – DNA ladder; Und – undigested; 1,3 – homozygous normal allele; 2,4,5 – heterozygous allele

CONCLUSION

Despite our study had some limitations like the impossibility to measure plasma levels of homocysteine and folates, our work provides data for the first time in a Romanian population group regarding risk factors for male infertility, possibly attributed to abnormal folate status.

Considering the fact that the genotype and allelic distribution of the MTHFR A1298C polymorphism observed in our study was similar in the two groups, we conclude that this polymorphism is not a genetic risk factor for male infertility in our Romanian population group. To better understand the etiology of male infertility, future studies will need to be conducted on more subjects to obtain a higher statistical significance and to focus on indentifying and studying new candidate genes in order to obtain a deeper understanding of the complex gene-to-gene and gene-nutrient interactions which have a profound effect on the studied pathology.
In the present study we evaluated for the first time the possibility of an association between the A1298C SNP in the MTHFR gene and male infertility in a Romanian population group. A few previous studies have evaluated the association of MTHFR C677T polymorphism in infertile patients from Germany, Netherlands, Italy, India, South Korea and China (24-30). Five of them (24,27-29) have reported an association between these polymorphisms in the MTHFR gene and male infertility. However, the MTHFR A1298C SNP has been studied less. Varinderpal et al. (2) reported no association between the A1298C SNP and male infertility in an Indian study group; while another study done on Chinese infertile men also (29) concluded that there is no association between this SNP and the idiopathic cases of male infertility.

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**CONCLUSION**

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MEHTYLENETERAHYDROFOLATE REDUCTASE A1298C POLYMORPHISM AND MALE INFERTILITY IN A ROMANIAN POPULATION GROUP

ACKNOWLEDGEMENTS

This study was partially supported by the research grant 546/2007, funded by the National Council for University Scientific Research, Ministry of Education, Research and Innovation, Romania.

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ACKNOWLEDGEMENTS

This study was partially supported by the research grant 546/2007, funded by the National Council for University Scientific Research, Ministry of Education, Research and Innovation, Romania.

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MALE FERTILITY DISORDERS

Infertility is a major health problem affecting 10-15% of couples seeking to have children and a male factor can be identified in about half of these cases. A significant proportion of infertile males are affected either by oligozoospermia (reduced sperm production) or azoospermia (lack of any sperm in the ejaculate).

The alteration of spermatogenesis can be the consequence of many causes but however 50% of infertile cases are unknown. Recently the growth of assisted reproduction techniques contributed to such research and the study of Y chromosome microdeletions into AZF regions shows their connection to non obstructive azoospermia; men with deletions in AZFa and AZFb regions, show severe defects in spermatogenesis, whereas deletions of AZFc region can be compatible with residual spermatogenesis.

The screening of AZF microdeletions results important to prevent their vertical transmission to sons in particular after the development of new reproduction techniques, that overcome spermatogenic defects and the biological selection.

Conventional PCR Kit  CE * IVD

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Reverse Hybridization CE * IVD

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<td>EES003020</td>
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METHIONINE SYNTHASE REDUCTASE (MTRR) GENE 66G>A POLYMORPHISM AS A POSSIBLE RISK FACTOR FOR RECURRENT SPONTANEOUS ABORTION

R.A. Popp, A.P. Trifa, Mariela Militaru, Tania Octavia Crisan, Felicia Petrisor, M.F. Farcas, F.A. Csernik, I.V. Pop

DEPARTMENT OF MEDICAL GENETICS, “IULIU HATIEGANU” UNIVERSITY OF MEDICINE AND PHARMACY, CLUJ-NAPOCA, ROMANIA

Summary

Low dietary folate intake has been blamed for many pathologies including cancers, thrombophilia, congenital anomalies (e.g. neural tube defects) and recurrent spontaneous abortions (RSA). An important biological function of folates, which is closely related to homocysteine levels, is to provide methyl groups, which are essential for DNA methylation reactions and for deoxynucleotide synthesis. There are many enzymes involved in the folate-homocysteine metabolic pathway and some polymorphisms in the genes coding for these enzymes can lead to an inappropriate activity of their corresponding enzymes. One of the key enzymes in the homocysteine metabolism is the methionine synthase reductase (MTRR) which catalyzes the regeneration of methylcobalamin, a cofactor for another important enzyme named methionine synthase (MTR). We hypothesized that the 66G>A substitution of the MTRR gene could be associated with an increased risk for recurrent spontaneous abortion (RSA). In a case-control study comprising 131 women with RSA and 131 healthy fertile women without any history of spontaneous abortion we genotyped MTRR 66G>A polymorphism by PCR-RFLP methods. We found that the MTRR variant GA and GA/AA genotypes were associated with a significant risk for RSA: odds ratio (OR), 2.00; 95% confidence interval (95% CI), 1.21-3.29 for GA and OR, 2.10; 95% CI, 1.28-3.43 for GA+AA compared with the GG genotype. In conclusion, our data provide evidence that there could be an association between MTRR 66G>A variant and RSA risk.

Key words: recurrent spontaneous abortion, MTRR gene, polymorphism, folate, homocysteine

Introduction

The main causes of recurrent spontaneous abortion (RSA) are known, but there are still a lot of unanswered questions regarding the etiology of pregnancy loses and thus many RSA remain unexplained. The folates levels are essential for fetal growth and development. Low folate and high homocysteine (Hcy) maternal levels have been associated with numerous pregnancy complications and a number of adverse pregnancy outcomes associated with placental insufficiency, such as fetal intrauterine growth restriction and fetal death (Pihusch et al., 2001). The metabolism of essential amino acids like methionine, histidine, serine, glycine is based on folate-dependent reactions (Parle-McDermott et al., 2005, Tamura and Picciano., 2006). Decreased folate or vitamin B12 levels resulting either from a deficient diet or a genetic predisposition increase the risk of spontaneous abortion. There are many key enzymes known to orchestrate the folate-homocysteine pathway. Some polymorphisms in the genes coding for these enzymes seem to be associated with an inappropriate folate and homocysteine metabolism. The most extensively studied genetic variant that...
influence folate and homocysteine metabolism is the methylenetetrahydrofolate reductase (MTHFR) gene polymorphism at nucleotide position 677 (677C>T), which leads to an alanine to valine substitution in the catalytic domain of the MTHFR enzyme. (Paz et al., 2002, Zetterberg et al., 2003, Forges et al, 2007). Methionine synthase reductase (MTRR) is another important enzyme that catalyzes the remethylation of homocysteine to methionine via a cobalamin and folate dependent reaction. MTRR has a crucial role to maintain cobalamin in an active form and is an important determinant of homocysteine concentration in plasma. The MTRR gene is located at 5p15.3-p15.2 and a common polymorphism in the MTRR gene, named 66G>A (formerly MTRR 66A>G) determine an amino acid substitution from methionine to isoleucine at codon 22 (p.M22I) (Zhang et al., 2005). The MTRR 66AA genotype seems to contribute to elevated homocysteine and low folate levels when is compared with the MTRR 66GG genotype (Gaughan et al., 2001). Because MTRR is involved in folate metabolism we attempt to investigate if the 66G>A polymorphisms of the MTRR gene could be associated with idiopathic RSA.

Material and methods
To search for the possible association between idiopathic RSA and MTRR 66G>A gene polymorphism we conducted a case-control study comprising a total number of 262 women. The RSA group consisted of 131 women with a history of at least two spontaneous abortions, declared idiopathic after possible causes of recurrent pregnancy losses were excluded by morphological, hormonal, chromosomal, infectious and immunological investigations. The control group comprised 131 healthy, fertile women, with at least one child and without any history of spontaneous abortion. The main characteristics of the two groups are shown in Table I. The study was conducted in accordance with The World Medical Association Declaration of Helsinki statements and written informed consent was obtained from each participant before genotyping procedures.

Table I . Demographic and pregnancy data of the study groups

<table>
<thead>
<tr>
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<th>RSA group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>131</td>
<td>131</td>
</tr>
<tr>
<td>Age</td>
<td>31 (18-44)</td>
<td>38 (32-65)</td>
</tr>
<tr>
<td>Pregnancy losses</td>
<td>3 (2-7)</td>
<td>0</td>
</tr>
</tbody>
</table>

For polymorphism testing, 5 ml of blood was drawn from each participant and collected in a tube with EDTA. 300 µl of blood was used for DNA extraction with Wizard Genomic DNA Purification Kit (Promega®, USA). For genotyping we used the PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) technique as previously described by Wilson (Wilson et al., 1999) with some modifications. The PCR reaction was set-up in a volume of 25µl reaction mix containing: 12.5µl 2xPCR MasterMix – Taq DNA-polymerase 0.05U/µl, MgCl₂ 4mM, dNTPmix 0.4mM each (Fermentas MBI®, Vilnius, Lithuania) 1µl 2mg/ml Bovine Serum Albumine solution, (Fermentas MBI®, Vilnius, Lithuania); 10 pmoles of each forward and reverse primers (Eurogentec®, Seraing, Belgium); approximately 50-100 ng genomic DNA; and nuclease-free water. The PCR conditions for amplification were: an initial denaturation at 94°C for 5 min; and then 35 cycles of denaturation 30 sec at 94°C, annealing 30 sec at 55°C and elongation 30 sec at 72°C, followed by a final elongation for 5 min at 72°C. The reaction was set up
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| Age | 31 (18-44) | 38 (32-65) |
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on a Mastercycler Gradient thermal cycler (Eppendorf®, Hamburg, Germany). A 12.5 µl aliquot of the amplicon solution was consequently digested overnight with 3 U of NdeI restriction enzyme (Fermentas MBI®, Vilnius, Lithuania), under the manufacturer digestion conditions. The digested fragments were resolved in a 3% MetaPhor agarose gel (Lonza®, Basel, Switzerland) stained with ethidium bromide and then visualised on a UV transilluminator (Vilber Lourmat®, Marne-la-Vallée, France). The observed alleles and genotypes frequencies were calculated for both groups and the Chi-square test for deviation was performed in order to establish if the genotypes distribution in the studied population were in Hardy-Weinberg equilibrium. A comparison of the results between the study group and control group was made and the differences were tested for significance through Chi-Square test using the EpiInfo 3.3.2 software (EpiInfo®, Atlanta, USA).

**Results and discussions**

The alleles and genotypes distribution in the two study groups can be analysed in Table II. The observed genotypes frequencies among the study groups were in agreement with Hardy-Weinberg equilibrium ($\chi^2 = 2.201$, $p = 0.137$). The genotypes frequencies of the MTRR 66G>A polymorphism were 51.14%, and 6.1% for the GA and AA genotype respectively, among the RSA group and 34.35% and 4.58%, respectively, among the control group. In the case of the homozygous AA genotype only, despite there is a difference between the RSA patients and control group, the level of statistical significance was not reached ($p>0.05$). This finding could be a consequence of the relative small size of the two groups studied. The difference regarding the distribution of heterozygous GA + homozygous AA genotypes in the RSA group versus control group was statistically significant (OR 2.10, 95%CI 1.28-3.43, $p<0.05$). Also, if we analyse the frequencies of the A allele in the two study groups, we can conclude that the A allele tends to be more frequent and could represent a risk factor in the RSA group (allele frequency 0.316 in the RSA group versus 0.217 in the control group; OR 1.66, 95%CI 1.12-2.46, $p = 0.013$).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>RSA group, n (%)</th>
<th>Control Group, n (%)</th>
<th>OR (95%CI)</th>
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<tr>
<td>GA</td>
<td>67 (51.14)</td>
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<td>AA</td>
<td>8 (6.1)</td>
<td>6 (4.58)</td>
<td>2.10 (1.28-3.43)</td>
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<tr>
<td>GA+AA</td>
<td>75 (57.3)</td>
<td>51 (38.9)</td>
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<tr>
<td>Allele frequencies</td>
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<tr>
<td>Total no. of alleles</td>
<td>262</td>
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<tr>
<td>G allele</td>
<td>0.683</td>
<td>0.782</td>
<td>1.66 (1.12-2.46)</td>
<td>0.013</td>
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<tr>
<td>A allele</td>
<td>0.316</td>
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In healthy individuals plasma homocysteine level and metabolism are well regulated but various environmental or genetic factors could determine an elevation of the levels of homocysteine (Sharma et al., 2006). Genetic variants of different genes coding for enzymes involved in the folate metabolism and homocysteine remethylation pathway might act as predisposing factors involved in abnormal fetal development and in pregnancy loss. One of the hypothesis is that the abnormal procoagulant activity of homocysteine in the presence of a low folate level has a
Genetic risk factors in infertile men with severe oligozoospermia and azoospermia


1Andrology Unit, Department of Urology, 2Department of Clinical Genetics and 3Department of Obstetrics and Gynaecology, Erasmus University Medical Centre, Rotterdam, The Netherlands
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BACKGROUND: Male infertility due to severe oligozoospermia and azoospermia has been associated with a number of genetic risk factors. METHODS: In this study 150 men from couples requesting ICSI were investigated for genetic abnormalities, such as constitutive chromosome abnormalities, microdeletions of the Y chromosome (AZF region) and mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. RESULTS: Genetic analysis identified 16/150 (10.6%) abnormal karyotypes, 8/150 (5.3%) AZFc deletions and 14/150 (9.3%) CFTR gene mutations. An abnormal karyotype was found both in men with oligozoospermia and azoospermia: 9 men had a sex-chromosomal aneuploidy, 6 translocations were identified and one marker chromosome was found. Y chromosomal microdeletions were mainly associated with male infertility, due to testicular insufficiency. All deletions identified comprised the AZFc region, containing the Deleted in Azoospermia (DAZ) gene. CFTR gene mutations were commonly seen in men with congenital absence of the vas deferens, but also in 16% of men with azoospermia without any apparent abnormality of the vas deferens. CONCLUSIONS: A genetic abnormality was identified in 36/150 (24%) men with extreme oligozoospermia and azoospermia. Application of ICSI in these couples can result in offspring with an enhanced risk of unbalanced chromosome complement, male infertility due to the transmission of a Y-chromosomal microdeletion, and cystic fibrosis if both partners are CFTR gene mutation carriers. Genetic testing and counselling is clearly indicated for these couples before ICSI is considered.

Key words: CFTR gene mutations/chromosomal abnormalities/ICSI/male infertility/Y chromosome microdeletions

Introduction

Male infertility has been associated with several genetic and non-genetic conditions, such as hypogonadotrophic hypogonadism, testicular maldescence, structural abnormalities of the male genital tract, genital infections, previous scrotal or inguinal surgery, varicoceles, chronic illness, medication and exposure to chemicals. In at least 40% of men no cause of the infertility was found (Crosignani et al., 1992). Genetic abnormalities were identified in men with unexplained oligozoospermia and azoospermia, including numerical and structural chromosomal abnormalities (Chandley, 1998), deletions of the azoospermia factor region (AZF) of the Y chromosome (Reijo et al., 1995; Vogt et al., 1996) and mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, commonly associated with congenital vas deferens abnormalities (Jaffe and Oates, 1994; Dohle et al., 1999). Most numerical chromosome abnormalities and AZF deletions are de-novo events in the parental germ cells. Some abnormalities associated with infertility are inherited, like reciprocal and Robertsonian translocations and CFTR mutations (Mak and Jarvi, 1996).

ICSI is the most significant recent development in the treatment of male infertility, enabling couples who were previously deemed infertile to produce offspring, however with the risk of passing on genetic abnormalities, and possibly decreased fertility. To assess the couple’s risk of transmitting a genetic abnormality we analysed the results of a combined andrological, cytogenetic and molecular genetic screening of 150 men with oligozoospermia and azoospermia. In addition, we discuss the necessity of genetic counselling for these infertile couples.

Materials and methods

For this study 150 men were selected from couples requesting ICSI who had an infertility duration of at least one year and a semen analysis showing <1x10⁹/ml motile sperm. History taking focused on urogenital development, chronic illness, pulmonary diseases, growth disturbances, medication, male accessory gland infections (MAGI), previous inguinal and scrotal surgery and occupational exposure to heat or chemicals. All men were investigated for genital malformations and ultrasound investigation of the scrotal content was
Genetic risk factors in infertile men with severe oligozoospermia and azoospermia

G.R.Dohle¹,⁴, D.J.J.Halley², J.O.Van Hemel², A.M.W.van den Ouweland², M.H.E.C.Pieters³, R.F.A.Weber¹ and L.C.P.Govaerts²

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BACKGROUND: Male infertility due to severe oligozoospermia and azoospermia has been associated with a number of genetic risk factors. METHODS: In this study 150 men from couples requesting ICSI were investigated for genetic abnormalities, such as constitutive chromosome abnormalities, microdeletions of the Y chromosome (AZF region) and mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. RESULTS: Genetic analysis identified 16/150 (10.6%) abnormal karyotypes, 8/150 (5.3%) AZFc deletions and 14/150 (9.3%) CFTR gene mutations. An abnormal karyotype was found both in men with oligozoospermia and azoospermia: 9 men had a sex-chromosomal aneuploidy, 6 translocations were identified and one marker chromosome was found. Y chromosomal microdeletions were mainly associated with male infertility, due to testicular insufficiency. All deletions identified comprised the AZFc region, containing the Deleted in Azoospermia (DAZ) gene. CFTR gene mutations were commonly seen in men with congenital absence of the vas deferens, but also in 16% of men with azoospermia without any apparent abnormality of the vas deferens. CONCLUSIONS: A genetic abnormality was identified in 36/150 (24%) men with extreme oligozoospermia and azoospermia. Application of ICSI in these couples can result in offspring with an enhanced risk of unbalanced chromosome complement, male infertility due to the transmission of a Y-chromosomal microdeletion, and cystic fibrosis if both partners are CFTR gene mutation carriers. Genetic testing and counselling is clearly indicated for these couples before ICSI is considered.

Key words: CFTR gene mutations/chromosomal abnormalities/ICSI/male infertility/Y chromosome microdeletions

Introduction

Male infertility has been associated with several genetic and non-genetic conditions, such as hypogonadotropic hypogonadism, testicular maldescence, structural abnormalities of the male genital tract, genital infections, previous scrotal or inguinal surgery, varicoceles, chronic illness, medication and exposure to chemicals. In at least 40% of men no cause of the infertility was found (Crosignani et al., 1992). Genetic abnormalities were identified in men with unexplained oligozoospermia and azoospermia, including numerical and structural chromosomal abnormalities (Chandley, 1998), deletions of the azoospermia factor region (AZF) of the Y chromosome (Reijo et al., 1995; Vogt et al., 1996) and mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, commonly associated with congenital vas deferens abnormalities (Jaffe and Oates, 1994; Dohle et al., 1999). Most numerical chromosome abnormalities and AZF deletions are de-novo events in the parental germ cells. Some abnormalities associated with infertility are inherited, like reciprocal and Robertsonian translocations and CFTR mutations (Mak and Jarvi, 1996).

ICSI is the most significant recent development in the treatment of male infertility, enabling couples who were previously deemed infertile to produce offspring, however with the risk of passing on genetic abnormalities, and possibly decreased fertility. To assess the couple’s risk of transmitting a genetic abnormality we analysed the results of a combined andrological, cytogenetic and molecular genetic screening of 150 men with oligozoospermia and azoospermia. In addition, we discuss the necessity of genetic counselling for these infertile couples.

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For this study 150 men were selected from couples requesting ICSI who had an infertility duration of at least one year and a semen analysis showing <1×10⁶/ml motile sperm. History taking focused on urogenital development, chronic illness, pulmonary diseases, growth disturbances, medication, male accessory gland infections (MAGI), previous inguinal and scrotal surgery and occupational exposure to heat or chemicals. All men were investigated for genital malformations and ultrasound investigation of the scrotal content was
biopsies from men carrying an AZF deletion exhibit a wide spectrum of spermatogenetic defects from complete absence of germ cells (Sertoli-cell-only syndrome) to maturation arrest with occasional production of mature, condensed spermatids (Reijo et al., 1995).

Men with defects of the Wolffian duct, presenting as CBAVD, were found to carry different mutations of the CFTR gene (Jaffe and Oates, 1994; Oates and Amos, 1994). CBAVD, previously described as a genitai form of cystic fibrosis, appears to be a heterogeneous clinical and genetic condition. We have shown that some patients with CBAVD also have non-genital symptoms of cystic fibrosis, like defective cellular chloride excretion and disturbed pancreatic function (Dohle et al., 1999). It has been suggested that CBAVD patients are compound heterozygotes for a severe mutation in one allele in combination with a mild CFTR gene mutation in the other. Alterations in the non-coding regions of the gene, such as the polypyrimidine stretch in intron 8, in combination with a mutation in the other allele, were found to cause abnormal levels of CFTR protein, due to exon 9 skipping translation (Chu et al., 1993). CFTR intron 8 DNA variants may alter the splicing efficiency of the CFTR mRNA in exon 9 (Kiesewetter et al., 1993), thus causing reduced concentrations of CFTR protein (Chillon et al., 1995). Impaired CFTR protein function may cause defective, but not absent chloride excretion resulting in absence of the vas deferens, but not in pulmonary or pancreatic insufficiency (Anguiiano et al., 1992). Mutations of the CFTR gene are commonly associated with obstructions of the male genital tract (Mak et al., 2000), and not with spermatogenetic failure (Pallaress-Ruiz et al., 1999). We detected at least one CFTR mutation in 4/6 men with CBAVD and in 10/144 men without a vas deferens-related problem. This seems to confirm the association of mutations in the CFTR gene with obstruction of the male genital tract rather than with primary testicular failure, although recently some men with CBAVD were found to have a defective spermatogenesis (Meng et al., 2001). However, the rate of mutations detected in this study in the non-obstructed group does seem elevated compared with the carrier risk in the Dutch population of 1:30 (De Vries et al., 1997). This prompted us to initiate an extended study with a larger cohort of patients. Since we tested previously for mutations that are common among cystic fibrosis patients, we are now using extensive screening methods to evaluate the true rate of CFTR mutations/variants in this group of infertile men.

In this study 38 genetic abnormalities were identified in 36 men from a population of 150 men with severe male infertility. The application of ICSI in these couples may lead to offspring with an enhanced risk of unbalanced chromosome complement, male infertility due to transmission of an AZF deletion, and cystic fibrosis in the case of related genital abnormalities. We conclude that all men with severe oligozoospermia and azoospermia should be offered genetic testing and counselling before assisted reproduction is applied.

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ARTICLE

Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders

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Received October 18, 1996; Revised and Accepted October 22, 1996 DBJ/EMBL/GenBank accession no U71285

Methionine synthase catalyzes the remethylation of homocysteine to methionine in a methylcobalamin-dependent reaction. We used specific regions of homology within the methionine synthase sequences of several lower organisms to clone a human methionine synthase cDNA by a combination of RT–PCR and inverse PCR. The enzyme is 1265 amino acids in length and contains the seven residue structure-based sequence fingerprint identified for cobalamin-containing enzymes. The gene was localized to chromosome 1q43 by the FISH technique. We have identified one missense mutation and a 3 bp deletion in patients of the cblG complementation group of inherited homocysteine/folate disorders by SSCP and sequence analysis, as well as an amino acid substitution present in high frequency in the general population. We discuss the possibility that a mild deficiency of methionine synthase activity could be associated with mild hyperhomocysteinemia, a risk factor for cardiovascular disease and possibly neural tube defects.

INTRODUCTION

Methionine synthase (EC 2.1.1.13, 5-methyltetrahydrofolate-homocysteine methyltransferase) catalyzes the remethylation of homocysteine to methionine in a reaction in which methylcobalamin serves as an intermediate methyl carrier. This occurs by transfer of the methyl group of 5-methyltetrahydrofolate to the enzyme-bound cob(II)alamin to form methylcobalamin with subsequent transfer of the methyl group to homocysteine to form methionine. Over time, cob(II)alamin may become oxidized to cob(I)alamin rendering the enzyme inactive. Regeneration of the functional enzyme occurs through the methionine synthase-mediated methylation of the cob(I)alamin in which S-adenosyl-L-methionine is utilized as methyl donor. In Escherichia coli, two flavodoxins have been implicated in the reductive activation of methionine synthase (1). A methionine synthase-linked reducing system has yet to be identified in mammalian cells.

Deficiency of methionine synthase activity results in hyperhomocysteinemia, homocystinuria and megaloblastic anemia without methylmalonic aciduria (2,3). Two classes of methionine synthase-associated genetic diseases have been proposed based on complementation experiments between patient fibroblast cell lines (4). One complementation group, cblE, has been postulated to be due to deficiency of the reducing system required for methionine synthase (5). Cells from patients in the cblE group fail to incorporate 14C-methyltetrahydrofolate into methionine in whole cells but have significant methionine synthase activity in cell extracts in the presence of a potent reducing agent. The cblG group is thought to be due to defects of the methionine synthase apoenzyme. Mutant cells from this group show deficient methionine synthase activity in both whole cells and cell extracts (4,6). Moreover, some cblG patients show defective binding of cobalamin to methionine synthase in cells incubated with radiolabelled cyanocobalamin (7).

The gene encoding methionine synthase has been cloned from several lower organisms, but not from mammals (Fig. 1). The cobalamin-dependent methionine synthase of E.coli has been crystallized and the structure of its active site determined (8,9). We used specific regions of homology within the methionine synthase sequences, including a portion of the cobalamin binding site determined from the E.coli enzyme, to design degenerate oligonucleotides for RT–PCR-dependent cloning of human methionine synthase. We confirm the identification of the cDNA sequences for human methionine synthase by the high degree of homology to the enzymes in other species and the identification of mutations in patients from the cblG complementation group.

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Associations of MTHFR DNMT3b 4977 bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylation of GSTM1 in non-obstructive infertility in Indian men

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Methylenetetrahydrofolate (MTHFR) and DNMT3b play imperative roles in DNA synthesis and de novo methylation. GSTM1 is involved in detoxification of carcinogens. Mitochondrial DNA deletion has been associated with lower motility in human sperm. We analysed if polymorphisms in MTHFR (C677T and A1298C) and DNMT3b (C46359T) are associated with non-obstructive male infertility. We also analysed if folate, vitamin B12, homocysteine (Hcy), 8-hydroxy-2’-deoxyguanosine (8-OHdG) levels, dietary folate intake and mtDNA deletion (4977 bp) affects fertility, such interactions are modified by deletion and methylation of GSTM1. In this case–control study, we included 179 oligoasthenoteratozoospermia patients and 200 fertile men. Single-nucleotide polymorphism analysis was performed by PCR-restriction fragment length polymorphism. The MTHFR (C677T and A1298C) and DNMT3b (C46359T) frequencies did not differ significantly in two groups. GSTM1 in association with mtDNA 4977 deletion is significantly associated with infertility. Plasma folate and vitamin B12 levels are decreased and total Hcy is elevated in infertile men. GSTM1 methylation status was investigated by methylation-specific PCR. Methylation is significantly correlated with GSTM1 reduced/loss of expression in infertile men. Infertile men have significantly higher 8-OHdG levels. Dietary folate intake is not linked with GSTM1 methylation. Low folate intake in association with CT + TT genotypes (C677T) has significant protective effect on GSTM1 methylation. Results indicate that micronutrients, 8-OHdG levels, mtDNA deletion and GSTM1 promoter methylation are frequent alterations in infertility.

Key words: CpG island hypermethylation/DNMT3b/GSTM1/infertility/mtDNA/MTHFR

Introduction

Male infertility in humans has been acknowledged as the cause of couple’s inability to bear children in 20–50% of total cases. Genetic causes such as Y chromosome microdeletions, translocation and chromosomal aberrations have been identified as risk factors for male infertility (Foresta et al., 2001; Dohle et al., 2002).

Folate is central to nucleotide synthesis and DNA methylation. Folate deficiency is known to occur frequently and related hyperhomocysteinaemia is considered a risk factor for many diseases, including infertility. Several nutrients and their metabolites can influence gene expression. DNA methylation (both maintenance and de novo mC) influences chromatin structure and gene expression. If transcription of a tumour suppressor or vital morphogenetic protein is repressed, it may contribute to oncogenesis and abnormal embryogenesis. Primary or secondary folate deficiency can result in aberrant DNA methylation. Folate deficiency (DNA lesions owing to low dNTP levels) can contribute to pathologies of various diseases (Rampersaud et al., 2000, Figure 1).

MTHFR is a key regulatory enzyme involved in folate metabolism, DNA synthesis and remethylation reactions. Homocysteine is a metabolite product of remethylation and trans-sulphuration reactions involving methionine. Total homocysteine (tHcy) levels could affect DNA synthesis and methylation. A common polymorphism in the MTHFR gene (C677T) results in a thermolabile phenotype associated with high levels of homocysteine. MTHFR gene mutation C677T could influence its biochemical activity and studies have shown that TT (mutant) has 30% activity when compared with CC. Similarly, A1298C polymorphism also reduces enzyme activity but to a lesser degree than C677T (Frosst et al., 1995; van der Put et al., 1998). Its deficiency could alter the synthesis of 5-methyl-tetrahydrofolate (5-MTHF), interrupt Hcy remethylation to methionine and cause hyperhomocysteinaemia. Folate deficiency-linked hyperhomocysteinaemia is a risk factor for many diseases including infertility. Recent studies have reported diverse associations between MTHFR C677T polymorphism and infertility (Bezold et al., 2001; Ebisch et al., 2003; Stuppia et al., 2003; Singh et al., 2005; Paracchini et al., 2006). Low folate coupled with MTHFR SNPs can alter RNA/DNA synthesis and has the potential to be linked with infertility (Stem et al., 2000). Animal model studies suggest, MTHFR plays a critical role in spermatogenesis due to exceptionally higher activity in adult testis than other organs (Chen et al., 2001).
DNMT3b (C46359T) gene, involved in folate metabolism and their associated risk for infertility. The prevalence of these genotypes were also evaluated in conjunction with GSTM1 genotype, 4977 bp deletion in mtDNA and micronutrients levels of folate, homocysteine and vitamin B12. This study showed that plasma folate level was associated with infertility risk. Our findings demonstrate relevance of folate metabolism in susceptibility to infertility among Indian male population. A few previous studies have evaluated the association of MTHFR C677T polymorphism in infertile patients.

**Figure 4.** (A) 5' Upstream sequence of GSTM1 gene (GeneBank accession No. NM_000561.2) showing the position of CpG island used for bisulphite sequencing and methylation-specific PCR (MSP). (B) Example of methylated CpG island in the patient. (C) Unmethylated CpG islands in control as determined by bisulphite sequencing (arrow position: CpG dinucleotide). (D) Percentage of non-detectable GSTM1 methylation in different genotypes of MTHFR and DNMT3b gene. (E) Representative examples of MSP. (F) Typical RT–PCR results of GSTM1 genes in infertile patients. GAPDH was used as an internal control. Expression of GSTM1 gene mRNAs was absent in samples 1, 3, 11 and 12 while samples 4, 6 and 9 show lower expression when compared with sample 7 in infertile patients.

**Table V.** Correlation in genotypes and GSTM1 methylation status in infertile group

<table>
<thead>
<tr>
<th>Gene</th>
<th>Methylation +ve</th>
<th>Methylation − ve</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTHFR C677T</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>27</td>
<td>30</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>11</td>
<td>38</td>
<td>0.32 (0.14–0.75)</td>
<td>0.0088*</td>
</tr>
<tr>
<td>TT</td>
<td>2</td>
<td>12</td>
<td>0.19 (0.04–0.75)</td>
<td>0.0331*</td>
</tr>
<tr>
<td>CT + TT</td>
<td>13</td>
<td>50</td>
<td>0.29 (0.13–0.64)</td>
<td>0.0034*</td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.81</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.19</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MTHFR A298T</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>25</td>
<td>35</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>13</td>
<td>40</td>
<td>0.46 (0.2–1.02)</td>
<td>0.0726</td>
</tr>
<tr>
<td>CC</td>
<td>2</td>
<td>5</td>
<td>0.56 (0.1–3.12)</td>
<td>0.69</td>
</tr>
<tr>
<td>AC + CC</td>
<td>15</td>
<td>45</td>
<td>0.47 (0.21–1.02)</td>
<td>0.08</td>
</tr>
<tr>
<td>Allele frequency</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.78</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.22</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DNMT3b C46359T</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>22</td>
<td>26</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>17</td>
<td>40</td>
<td>0.5 (0.23–1.12)</td>
<td>0.1074</td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>14</td>
<td>0.08 (0.01–0.69)</td>
<td>0.0058*</td>
</tr>
<tr>
<td>CT + TT</td>
<td>18</td>
<td>54</td>
<td>0.39 (0.18–0.86)</td>
<td>0.029*</td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.76</td>
<td>0.575</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.24</td>
<td>0.425</td>
<td></td>
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</tbody>
</table>

*Significant at P < 0.05.

**Table VIA.** Odds ratio (OR) for GSTM1 methylation and dietary folate intake

<table>
<thead>
<tr>
<th>Dietary folate intake</th>
<th>Methylation − ve</th>
<th>Methylation + ve</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>65</td>
<td>17</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>Low</td>
<td>74</td>
<td>23</td>
<td>1.19 (0.58–2.42)</td>
</tr>
</tbody>
</table>
It has already been shown that sperm concentration is significantly increased by folic acid and zinc sulphate treatment from fertile and subfertile men with CC genotype \((MTHFR)\) but not from CT and TT genotype (Ebisch et al., 2003).

It has been established that oxidative stress can cause DNA damage in human sperm and can cause infertility (Hideya et al., 1997). In this study we found significantly higher 8-OHdG levels in infertile men than fertile men, when analysed in relation to \(GSTM1\) genotype and mtDNA 4977 bp deletion. These observations give further support to the notion that sperm 8-OHdG levels may act as a good marker of oxidative stress in testis and their ability to cause potential damage to sperm DNA and their ability to induce fertilization. Oxidative stress can cause folate depletion. There are two mechanisms that can explain the occurrence of oxidative folate cleavage. First, oxidation of pteridine ring leads to dihydrofolate and then folic acid thereby retaining folate as an active metabolite (Loscalzo, 1996). It is, therefore, anticipated that its significantly increased by folic acid and zinc sulphate treatment from fertile and subfertile men with CC genotype \((MTHFR)\) but not from CT and TT genotype (Ebisch et al., 2003).

It has been established that oxidative stress can cause DNA damage in human sperm and can cause infertility (Hideya et al., 1997). In this study we found significantly higher 8-OHdG levels in infertile men than fertile men, when analysed in relation to \(GSTM1\) genotype and mtDNA 4977 bp deletion. These observations give further support to the notion that sperm 8-OHdG levels may act as a good marker of oxidative stress in testis and their ability to cause potential damage to sperm DNA and their ability to induce fertilization. Oxidative stress can cause folate depletion. There are two mechanisms that can explain the occurrence of oxidative folate cleavage. First, oxidation of pteridine ring leads to dihydrofolate and then folic acid thereby retaining folate as an active cofactor. Second, reduced folates are particularly prone to oxidative scission thus rendering the product metabolically inactive as a cofactor (Suh et al., 2001). This can lead to hyperhomocysteinaemia. High levels of homocysteine can lead to auto-oxidation through the production of hydrogen peroxide \((H_2O_2)\)—a harmful reactive oxygen metabolite (Loscalzo, 1996). It is, therefore, anticipated that its increased production may be associated with Hcy-mediated DNA damage. Hcy increases intracellular \(H_2O_2\) generation, inducing apoptosis as a result of severe DNA damage. Oxidative stress induces peroxidative damage in sperm’s plasma membrane and damage in mitochondrial and nuclear DNA (Aitken and Krausz, 2001). There is evidence to suggest that \(H_2O_2\) treatment causes more damage in asthenozoospermic infertile men than in normozoospermic infertile and fertile men (Hughes et al., 1996). Hyperhomocysteinaemia leading to precocious atherosclerosis of testicular arteries could be one mechanism behind \(MTHFR\) polymorphism and infertility.

### Table VII. Joint effects of genotype and folate intake on \(GSTM1\) methylation

<table>
<thead>
<tr>
<th>Gene mutation</th>
<th>Folate intake</th>
<th>(GSTM1) methylation</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MTHFR 677)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>High</td>
<td>17</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>CC</td>
<td>Low</td>
<td>13</td>
<td>1.9 (0.66–5.46)</td>
</tr>
<tr>
<td>CT and TT</td>
<td>High</td>
<td>13</td>
<td>0.71 (0.21–2.44)</td>
</tr>
<tr>
<td>CT and TT</td>
<td>Low</td>
<td>37</td>
<td>0.29 (0.1–0.89)*</td>
</tr>
<tr>
<td>(MTHFR 1298)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA and CC</td>
<td>High</td>
<td>20</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>AA and CC</td>
<td>Low</td>
<td>25</td>
<td>0.91 (0.28–2.95)</td>
</tr>
<tr>
<td>AC</td>
<td>High</td>
<td>18</td>
<td>1.59 (0.49–5.05)</td>
</tr>
<tr>
<td>AC</td>
<td>Low</td>
<td>17</td>
<td>2.52 (0.83–7.62)</td>
</tr>
<tr>
<td>DNA135b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>High</td>
<td>14</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>CC</td>
<td>Low</td>
<td>12</td>
<td>0.78 (0.247–2.54)</td>
</tr>
<tr>
<td>CT and TT</td>
<td>High</td>
<td>32</td>
<td>0.4 (0.14–1.13)</td>
</tr>
<tr>
<td>CT and TT</td>
<td>Low</td>
<td>9</td>
<td>0.48 (0.16–1.43)</td>
</tr>
</tbody>
</table>

*Significant at \(P < 0.05\).

![Figure 5. 8'-Hydroxy-2'-deoxyguanosine (8-OHdG) levels in sperm DNA in fertile and infertile men. *Significant at \(P < 0.001\); **significant at \(P < 0.05\).*](http://molehr.oxfordjournals.org/)

### Table VII. Hormone concentration in fertile and infertile groups

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Fertile median (range)</th>
<th>Infertile median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (nM)</td>
<td>22.5 (6.9–34.6)</td>
<td>21.3 (7.2–31.8)</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>3.2 (1.4–6.2)</td>
<td>3.5 (1.7–9.8)</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>3.3 (1.8–7.4)</td>
<td>3.5 (1.2–10.4)</td>
</tr>
<tr>
<td>SHBG (nM)</td>
<td>31.1 (17.2–45.3)</td>
<td>28.1 (20.1–58.2)</td>
</tr>
<tr>
<td>E2 (pM)</td>
<td>79.6 (49.0–116.0)</td>
<td>82.1 (55.0–131.0)</td>
</tr>
</tbody>
</table>

FSH: follicle stimulating hormone; LH: luteinizing hormone; SHBG: sexual hormone binding globulin; E2: estradiol; T: testosterone.
Absence of a functional copy of *GSTM1* and increased Hcy levels together can cause sperm membrane damage and also induce apoptosis. Serum folate and vitamin B12 levels were also tested because of their strong association to homocysteine metabolism, in terms of a possible therapeutic role. Although serum folate and vitamin B12 levels were lower in infertile men when compared with fertile men, these differences did not reach statistical significance. Yet, our data clearly show that hyperhomocysteinemia is more frequent in infertile than fertile men and can be regarded as one of the factors associated with infertility.

Epigenetic alterations in DNA without concomitant changes in underlying genetic code are now known to occur frequently in various human diseases. Our data also unveil, for the first time, that promoter hypermethylation of *GSTM1* is a common event occurring in the testis of infertile men, with 40 of 120 showing such epigenetic aberrations. In this study, *GSTM1* was shown to be expressed in human normal testicular tissue. It is under-expressed (≥2-fold drop of level in matched normal tissue) in 47% of infertile men. It is a frequent abnormality in infertile men and it may have a significant role in causing infertility. Our results are in line with a previous report (Jhaeveri et al., 2001) suggesting the decreased expression of H-cadherin by decreased intracellular folate is associated with hypermethylation of CpG islands. It is anticipated that *GSTM1* repression under folate-deficient conditions may act as an effector of infertile phenotype having low folate levels. We have shown for the first time that *GSTM1* promoter methylation is associated with low dietary folate intake in infertile men. This may occur either as a direct or indirect consequence of altered folate intake. Folate is a major methyl donor for DNA methylation. It provides substrate for *MTHFR* to convert 5,10-methylene-tetrahydrofolate (5,10-MTHF) to 5-MTHF subsequently metabolized to methionine (Ross, 2003). Alteration in methylation maintenance may be causally associated with global hypomethylation of the genome. Our data supports the notion that infertility risk posed by decreased dietary folate intake might in part be attributable to enhanced concurrent promoter hypermethylation in *GSTM1* gene.

Localized folate diminution could result in a transformational change in testicular tissue. To compensate for the high demands of folates required for cell proliferation, folate receptors might be up-regulated. Under such conditions, TT genotype, associated with greater enzyme sensitivity to reduced availability of 5-MTHF, would maintain the required supply of 5,10-MTHF for nucleotide synthesis. However, CC or CT genotype having delayed inhibition due to its greater stability of *MTHFR* enzyme complex at lower concentrations of 5-MTHF could result in a compromised supply of single carbon units for the methylation of dUMP to dTMP. This could lead to increased DNA damage owing to reduced availability of 5,10-MTHF for DNA synthesis. This could lead to uracil misincorporation and DNA strand breaks. Dysregulation of methylation maintenance may be casually associated with global DNA hypomethylation. Alternatively, infertility risk due to low dietary folate intake may in part be attributed to the enhanced occurrence of concurrent hypermethylation in the promoter region of specific genes including *GSTM1* as seen in the present study. Genetic and environmental factors are likely to be important in determining CpG-island methylation levels. CpG island methylation in *GSTM1* gene showed significantly less methylation in *DNMT3b* TT homozygotes when compared with CC/CT genotype. *DNMT3b* mediates *de novo* DNA methylation, and *C/T* transition has been shown to increase 30% *in vitro* transcriptional activity (Shen et al., 2002). However, lower methylation levels of *GSTM1* gene observed in *DNMT3b* TT homozygotes are contrary to expectations and could influence global DNA methylation independently and/or hypermethylation of CpG islands in promoter regions of various genes including *GSTM1* as shown here. From our results we can conclude that TT mutants (*DNMT3b*) show significantly less methylation when compared with CC. We have found that a correlation exists between *GSTM1* methylation and CC genotype of *MTHFR* C677T, thus establishing the fact that *MTHFR* enzyme integrity promotes DNA methylation. Our results support the hypothesis that genetic factors affecting function of *DNMT3b* and *MTHFR* genes, and low folate intake in CC (*MTHFR*) individuals may trigger the tendency of acquiring aberrant CpG island methylation in *GSTM1* gene in infertile men.

Sperm chromatin structure and DNA integrity are known to have a critical influence on the fertilization process. Infertile men are found to have a higher fraction of sperm with chromatin defects and DNA breaks than fertile controls. Oxidative stress is suggested as one of the factors responsible for DNA damage in ejaculated sperm. Morphologically, abnormal spermatozoa and leukocytes are the main source of excess ROS generation in semen. Mechanisms like defective sperm chromatin packaging, chaotic apoptosis and oxidative stress can cause sperm damage. Therefore, in light of this *GSTM1* polymorphisms and methylation, and mtDNA deletions might be assessed to determine the impact of these factors on infertility. Errors in meiotic pairing and recombination have also been suggested to play a role in male infertility. An increase in sex chromosomal aneuploidy could be related to higher susceptibility of XY bivalent to flawed segregation patterns as a result of smaller homologous regions available for synapsis and recombination (i.e. pseudo-autosomal region). Infertility can arise due to abnormalities in terminal differentiation (post-recombination) of germ cells. These factors along with the various parameters studied here in detail should be investigated in infertile men attending the fertility clinics.

The findings from the present study indicate that decreased levels of folate, B12 and increased levels of tHcy and 5-OHDG are associated with increased infertility risk. Similarly, an epigenetic change in *GSTM1* methylation leading to its silencing along with its loss/reduced expression further aggravates the infertility risk. The present study showed positive association between mtDNA deletion and infertility. However, we could not find significant association among *MTHFR*, *DNMT3b* and *GSTM1* polymorphisms and infertility.

### References

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