Methylenetetrahydrofolate reductase C677T and A1298C variants do not affect ongoing pregnancy rates following IVF


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BACKGROUND: There is concern that IVF could compromise normal imprinting and methylation of DNA. Methylenetetrahydrofolate reductase (MTHFR) regulates the flow of folic acid-derived, one-carbon moieties for methylation and is critical to early embryonic development. Therefore, we hypothesized that common polymorphisms in MTHFR could associate with IVF outcome. METHODS: MTHFR C677T and A1298C polymorphism genotyping was performed on 374 subjects for this study, representing 197 couples undergoing IVF in a university setting from July 2005 to January 2006. Analysis of variance (ANOVA), chi-square and/or multivariate analyses were used to assess whether these polymorphisms are associated with embryo quality or with ongoing pregnancy or spontaneous abortion rates.

RESULTS: Allele frequencies for C677T (p = 0.67, q = 0.33) and A1298C (p = 0.71, q = 0.29) were in Hardy–Weinberg equilibrium. The C677T and A1298C variants, either alone or in combination, did not associate with embryo quality or short-term pregnancy outcome. CONCLUSIONS: The common polymorphisms in MTHFR are not associated with embryo quality, as defined by cell number or fragmentation score, or with short-term pregnancy outcomes. Therefore, in our population in which women receive adequate folic acid, MTHFR genotypes are not informative in explaining IVF failure. Further studies, however, examining birth outcomes and the other enzymes in the folic acid pathway are warranted.

Key words: methylenetetrahydrofolate reductase/MTHFR/IVF/folic acid/pregnancy rate

Introduction

Complex epigenetic processes, which change the methylation status of the genome, are fundamental to mammalian gene regulation (Razin and Shemer, 1995) and are critical to early embryonic development (Khosla et al., 2001; Shi and Haaf, 2002). Methyl groups, passed from folic acid through a series of enzymes, contribute to the production of S-adenosylmethionine (SAM), the ultimate methyl donor that is involved in hundreds of biologic transmethylation reactions. As such, folic acid is indispensable for embryonic development (Luccock, 2000). An enzyme critical to the folic acid pathway is methylenetetrahydrofolate reductase (MTHFR). Deficiencies of folic acid or defects in MTHFR have demonstrated DNA hypomethylation and abnormal biochemical and/or phenotypic changes in model animals (Kim et al., 1997; Chen et al., 2001), cell culture (Duthie et al., 2000; Kimura et al., 2004) and humans (Jacob et al., 1998; Stern et al., 2000; Friso et al., 2002; Castro et al., 2004).

Abnormalities in methylation during preimplantation embryogenesis could have immediate or even long-term developmental consequences. Several studies suggest that aberrant methylation, possibly due to the culture environment, leads to loss of imprinting and/or changes in gene expression. For example, in mice, loss of imprinting at the H19 paternal allele occurs when the embryos are cultured in Whitten’s medium, a very simple embryo culture medium, but not when cultured in the more complex synthetic oviductal medium enriched with potassium (KSOM) supplemented with amino acids (Doherty et al., 2000). Such changes could result in abnormal growth regulation (Young et al., 1998, 2001; De Rycke et al., 2002). In humans, two imprinting disorders have been described following IVF, Angelman syndrome (Cox et al., 2002; Orstavik et al., 2003) and Beckwith–Weideman syndrome (DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003), and in both, the alterations are predominantly hypomethylation of imprinted genes (Niemitz et al., 2004); this is in stark contrast to non-IVF children with the disorders, in which the majority are genetically, not epigenetically, determined.

MTHFR has well-characterized polymorphisms that affect its activity (reviewed in Robien and Ulrich, 2003). The mutation at C677T creates a thermolabile enzyme with only 30% of wild-type activity, whereas that at A1298C affects the SAM-regulatory domain and has ~60% of wild-type activity (Kang et al., 1998; van der Put et al., 1998; Guenther et al., 1999). Although mutations in MTHFR have been associated with many developmental abnormalities and pregnancy loss (Nelen et al., 2004; Khosla et al., 2001; Shi and Haaf, 2002).
et al., 1998; Isotalo et al., 2000; Zetterberg et al., 2002), its role in fertility has not been vigorously studied. Azem et al. (2004) demonstrated an increase in the incidence of the MTHFR 677 TT mutant genotype, albeit not significant, in women failing at least four cycles of IVF, and recently, Haggarty et al. (2006) demonstrated that women undergoing IVF and carrying the MTHFR 1298 CC mutant genotype were less likely to become pregnant than those with the wild-type 1298 AA genotype. Our previous findings have demonstrated that MTHFR is expressed in human oocytes and preimplantation embryos; therefore, we hypothesized that mutations in MTHFR could compromise embryo viability during IVF, in which they are routinely cultured in media that lack methyl donors, resulting in decreased ongoing pregnancy rates.

Materials and methods

Study subjects

Between July 2005 and January 2006, 374 patients, partners or their donors presenting to the UCSF Center for Reproductive Health for IVF, representing 197 embryo transfer cycles, were recruited into this study. There were no initial exclusion criteria. No participants actively withdrew from the study; however, in some instances, samples were not available from the male partners. All female patients were counselled to take prenatal vitamins containing at least 400 mcg folic acid during their initial pre-pregnancy counselling visit. This study was approved by the UCSF Committee on Human Research.

Sample collection

Each study participant had a cheek swab performed for DNA collection. Cheek swabs were collected from the inner cheek using a plastic 1-μl Sterilloop (Fisher Scientific, Pittsburgh, PA, USA), and the loop portion was cut off taking care to avoid contamination and then placed in a 0.2-ml PCR tube (E&K Scientific, Campbell, CA, USA) for storage at −80°C until processing. For those cases in which an anonymous male donor was used and a cheek swab was unavailable, 5 μl of sperm remaining after the procedure was collected and stored for genotyping analysis.

Each sample was subsequently treated with 5 μl of lysis buffer [200 mM KOH and 50 mM diethiothreitol (DTT)], vortexed and pulse centrifuged and then neutralized with 5 μl of neutralization buffer (900 mM Tris–HCl, pH 8.3, and 200 mM HCl). All reagents were from Sigma (St Louis, MO, USA). The samples were subsequently diluted to a final volume of 200 μl and stored at −20°C.

Genotype determination

Taqman SNP genotyping assays for the two common polymorphisms in MTHFR have previously been used successfully (Ulvik and Taqman SNP genotyping assays for the two common polymorphisms CCG-NFQ; A1298C forward primer, 5′-VIC dye-ATGAAATCGGCTCCCGC-NFQ (non-fluorescent quencher); C677T reporter ‘C’, 5′-GAGGAGGACGTCGAAAGATG; C677T reporter ‘T’, 5′-FAM dye-ATGAAATCGACTCAAAAGAAAATCGCTGATG; C677T reporter ‘A’, 5′-VIC dye-ATGAAATCGACTCAGGAGGTAAAGA; C677T reporter ‘G’, 5′-FAM dye-CAGTGAAGCTTAAAGGTGTC-NFQ; and A1298C reporter ‘C’, 5′-VIC dye-CAGTGAAGCTTAAAGGTGTC-NFQ. Taqman reactions were performed according to manufacturer’s instructions in an iCycler (Bio-Rad, Hercules, CA, USA) using 5 μl of DNA sample prepared as mentioned above in a 20-μl reaction with Taqman Universal PCR Master Mix (Applied Biosystems). The reactions were performed in 96-well plates (Bio-Rad) covered with 15 μl of mineral oil (Sigma) and optical tape (Bio-Rad).

The raw data for each cycle of PCR performed, for both the VIC and the FAM reporters, were copied to a Microsoft Excel spreadsheet. These data were transformed using Visual Basic code for immediate upload into a Microsoft Access database to eliminate errors and analyse the resulting genotypes. The data were stored and plotted for each sample in Microsoft Access for both dye layers simultaneously, therefore providing visual analysis of the genotype, which was determined in an unbiased fashion without regard to clinical outcome. Representative genotype plots are shown in Figure 1.

Statistical analysis

Initial comparisons of subject and embryo characteristics were performed using analysis of variance (ANOVA). The ongoing pregnancy rate was the primary outcome variable, which was assessed independently for the genotype of the women and men. The combined effects of the genotypes (677/1298) were assessed categorically and ordered 1–6 (WW, WH, HW, WM, WM, MW, and M, where W = wild type, H = heterozygous and M = mutant) based on their presumed enzymatic activity as determined in vitro (van der Put et al., 1998; Weisberg et al., 1998; Guenther et al., 1999). Multivariate regression analyses were performed to adjust for the following potential confounders: female age, parity, the number of oocytes retrieved and the number of embryos transferred. These variables were selected because of their established association with the outcomes. Goodness-of-fit test and tests for linear trend were done to determine whether the combined effect of the genotype demonstrated a linear trend. Chi-square tests were used for univariate comparisons to assess spontaneous abortion rate, as too few samples were available to use multivariate regression analysis.

Tests were done to determine whether the genotypes were associated with embryo growth (cell number) and day 3 fragmentation. All embryos created were included in the analyses. To account for the clustered nature of the data (repeat measures within the same individual), we performed generalized estimated linear equations. All data were analysed in Stata version 7.0 (Stata Corporation, College Station, TX, USA). Tests were declared statistically significant for a two-sided P value <0.05.

Embryo growth was scored by the number of cells on day 3 of development. Fragmentation was scored as 1, no fragmentation; 2, <10% fragmentation; 3, <25% fragmentation; 4, <50% fragmentation and 5, ≥50% fragmentation. A positive pregnancy test was defined as having a serum βhCG > 2 IU/l 14 days following embryo transfer. A clinical pregnancy was defined as having a sac on initial ultrasound examination at ~6 weeks’ gestation. Ongoing pregnancy was defined as having at least one intrauterine gestation with cardiac activity progressing into the second trimester.

Results

Genotype determination

Of the 374 samples collected in which outcome data were available for this study, 368 (98.4%) gave clear genotyping results at both loci. Two samples (0.5%) were indeterminate at the 1298 locus despite repeat testing; however, we were reasonably sure of the four (1.1%) other genotypes (two wild type and two heterozygous). Five patients, whose samples were collected in duplicate during different IVF cycles, gave identical results using this testing method. In addition, several patients had commercial evaluation of their genotype for clinical
reasons, and this method of testing was concordant in all cases (14/14 for C677T and 8/8 for A1298C). Of the 374 samples, 197 came from women (who contributed oocytes) and 177 came from men (who contributed the sperm); the difference of 20 samples was due to the male partner being unavailable for sample collection. In our population of patients undergoing assisted reproduction, the C677T allele frequencies ($p = 0.67$, $q = 0.33$) and A1298C allele frequencies ($p = 0.71$, $q = 0.29$) were similar to the published literature (Botto and Yang, 2000; Robien and Ulrich, 2003) and were in Hardy–Weinberg equilibrium.

**Effect of MTHFR gene variants C677T and A1298C IVF outcome**

Because MTHFR is expressed in oocytes, we expected it to function during the first few cell divisions before the activation of the male genome. Therefore, we examined the effect of the female MTHFR genotype on ongoing pregnancy rates following IVF. Of the women who contributed oocytes for this study, 197 had genotypic data for C677T and 196 for A1298C (Table I). Because the male genome becomes transcriptionally active between the 4- and 8-cell stage of development (Braude et al., 1988; Dobson et al., 2004), we also tested whether the genotype of the men contributing sperm influenced the above outcomes. Therefore, in those cycles in which genotypic data were available for the women who contributed oocytes and for the men who contributed sperm, 177 had genotypic data for C677T and 176 had genotypic data for A1298C (Table II). We also examined the combined effect of the C677T and A1298C mutations (Table III), their order being based on their presumed enzymatic activity as determined *in vitro* (van der Put et al., 1998; Weisberg et al., 1998; Guenther et al., 1999).

We first examined whether any of the genotypes associated with embryo quality, as determined by cell number and day 3 fragmentation. Although tests for significance among the embryos created demonstrated some significant differences for embryo cell number and day 3 embryo fragmentation using ANOVA (Tables I and II), the differences disappeared after using generalized estimated linear equations to account for the clustered nature of the data (Tables I and II; adjusted $P$ values).

Chi-square tests were performed on the raw data for ongoing pregnancy rates and spontaneous abortion rates among the different MTHFR genotypes of the women and men in the study. None demonstrated significance. Because the age of the women contributing oocytes is exceedingly important in determining IVF outcome and there were some significant differences in female age among the various MTHFR genotypes (Tables I and III), we further performed multivariate regression analysis. After adjusting for age of the women contributing oocytes, parity, the number of oocytes retrieved and the number of embryos transferred, none of the genotype combinations (for either the women or the men) significantly affected ongoing pregnancy rates. There was also no effect on positive pregnancy test or clinical pregnancy rate. Because of the small number of spontaneous abortions, it was not possible to perform multivariate regression analysis on this outcome. Tests for a trend of functional enzymatic activity among the six genotype combinations for C677T and A1298C for the women or the men were also not significant (Table III). Figure 2 shows the adjusted odds ratios for ongoing pregnancy among the different genotype combinations.

Finally, we determined in all crosswise comparisons whether the female genotype had an independent effect on ongoing pregnancy while controlling for the male genotype.
Table I. Characteristics and cycle outcomes based on the female methylenetetrahydrofolate reductase (MTHFR) genotype

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Heterozygous</th>
<th>Mutant</th>
<th>( P ) value(^\dagger )</th>
<th>Adjusted ( P ) value(^\dagger )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female 677</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of transfer cycles = 197 (100%)</td>
<td>96 (48.7%)</td>
<td>86 (43.7%)</td>
<td>15 (7.6%)</td>
<td>Not significant</td>
<td></td>
</tr>
<tr>
<td>Average female age ± SD</td>
<td>35.5 ± 5.4</td>
<td>34.7 ± 5.3</td>
<td>37.9 ± 5.5</td>
<td>Not significant</td>
<td></td>
</tr>
<tr>
<td>Average transfer number ± SD</td>
<td>2.9 ± 1.4</td>
<td>2.7 ± 1.1</td>
<td>3.5 ± 1.5</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>Number of embryos = 1546 (100%)</td>
<td>749 (48.4%)</td>
<td>658 (42.6%)</td>
<td>139 (9%)</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>Average cell number ± SD</td>
<td>7.0 ± 2.0</td>
<td>6.9 ± 2.1</td>
<td>6.8 ± 2.1</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>Average fragmentation ± SD</td>
<td>2.7 ± 1.2</td>
<td>2.6 ± 1.3</td>
<td>2.8 ± 1.3</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>Pregnancy rate = 92/197 (46.7%)</td>
<td>46 (47.9%)</td>
<td>41 (47.7%)</td>
<td>5 (33.3%)</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>SAb rate = 24/116 (20.7%)</td>
<td>11/57 (19.3%)</td>
<td>11/52 (21.2%)</td>
<td>2/27 (28.6%)</td>
<td>Not significant</td>
<td>Not determined</td>
</tr>
<tr>
<td>Female 1298</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of transfer cycles = 196(^a) (100%)</td>
<td>96 (49.0%)</td>
<td>78 (39.8%)</td>
<td>22 (11.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average female age ± SD</td>
<td>36.4 ± 4.9</td>
<td>35.2 ± 5.4</td>
<td>32.6 ± 6.9</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>Average transfer number ± SD</td>
<td>2.9 ± 1.4</td>
<td>2.9 ± 1.1</td>
<td>2.9 ± 1.5</td>
<td>Not significant</td>
<td></td>
</tr>
<tr>
<td>Number of embryos = 1537 (100%)</td>
<td>783 (50.9%)</td>
<td>588 (38.3%)</td>
<td>166 (10.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average cell number ± SD</td>
<td>6.9 ± 2.0</td>
<td>6.9 ± 21</td>
<td>7 ± 2.1</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>Average fragmentation ± SD</td>
<td>2.8 ± 1.2</td>
<td>2.6 ± 1.2</td>
<td>2.8 ± 1.2</td>
<td>0.017</td>
<td>Not significant</td>
</tr>
<tr>
<td>Pregnancy rate = 92/196 (46.9%)</td>
<td>41 (42.7%)</td>
<td>38 (48.7%)</td>
<td>13 (59.1%)</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>SAb rate = 23/115 (20.0%)</td>
<td>16/57 (28.1%)</td>
<td>6/44 (13.6%)</td>
<td>1/14 (7.1%)</td>
<td>Not significant</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

\(^\dagger\)Analysis of variance (ANOVA) for age, transfer number, cell number and fragmentation; \( \chi^2 \) for ongoing pregnancy and spontaneous abortion (SAb) rates.

and adjusting for all of the above variables. This ‘inferred’ embryonic genotype had no effect on any of the outcome parameters, and the test for trend of functional enzymatic activity was also not significant.

Discussion

In order for our hypothesis to be plausible, it was important to demonstrate that MTHFR was expressed during preimplantation development. Our previous microarray data (Dobson et al., 2004) demonstrated that MTHFR transcripts were indeed present in human oocytes and preimplantation embryos. Subsequent confirmation of MTHFR gene expression was performed by real-time PCR (unpublished data). These studies provided impetus to further examine our hypothesis.

Our results demonstrate that the MTHFR gene variants do not affect IVF outcomes, in which embryos are cultured in media deficient in folic acid, methionine and SAM. This is somewhat surprising, given the large body of evidence demonstrating that folate deficiency, especially in the presence of mutations of MTHFR, causes hypomethylation of DNA and that proper DNA methylation is critical to normal embryonic growth and development. In this study, the MTHFR polymorphisms were not associated with pregnancy, embryo quality or the frequency of early losses before or after the documentation of fetal cardiac activity in this IVF population (Tables I–III, Figure 2).

Although our results do not implicate MTHFR C677T or A1298C in failed ongoing pregnancies following IVF, our hypothesis stands. We postulate that environmental and genetic
### Table III. Characteristics and cycle outcomes based on the female or male combined C677T and A1298C methylenetetrahydrofolate reductase (MTHFR) genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Female 677/1298</th>
<th>Male 677/1298</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type/wild type</td>
<td>Wild type/heterozygous</td>
</tr>
<tr>
<td>Number of transfer cycles</td>
<td>196 (100%)</td>
<td>176 (100%)</td>
</tr>
<tr>
<td>Average female age ± SD</td>
<td>37 ± 4.2</td>
<td>36.5 ± 5.3</td>
</tr>
<tr>
<td>Average transfer number ± SD</td>
<td>3.1 ± 1.6</td>
<td>3.1 ± 1.7</td>
</tr>
<tr>
<td>Number of embryos</td>
<td>271 (17.6%)</td>
<td>197 (11.8%)</td>
</tr>
<tr>
<td>Average cell number ± SD</td>
<td>7.0 ± 2.9</td>
<td>6.7 ± 2.1</td>
</tr>
<tr>
<td>Average fragmentation ± SD</td>
<td>2.7 ± 1.1</td>
<td>2.6 ± 1.2</td>
</tr>
<tr>
<td>Pregnancy rate</td>
<td>92/196 (46.9%)</td>
<td>81/176 (46.0%)</td>
</tr>
<tr>
<td>SAb rate</td>
<td>3/19 (15.8%)</td>
<td>12/14 (13.4%)</td>
</tr>
</tbody>
</table>

1. Analysis of variance (ANOVA) for age, transfer number, cell number and fragmentation; \( \chi^2 \) for ongoing pregnancy spontaneous abortion (SAb) rates.
2. Multivariate analysis; \( P \leq 0.05 \) for significance in all tests.
in oocytes as determined by microarray analysis (data not shown). It is known that folic acid is present in follicular fluid, and its supplementation decreases serum and follicular fluid homocysteine levels and is associated with better quality and more mature oocytes (Szymanski and Kazdepeka-Zieminska, 2003). Although we did not test for elevated homocysteine levels, all of our infertile patients were taking prenatal vitamins, and previous studies have shown a reduction in homocysteine levels in women who supplement their diets with folic acid, regardless of MTHFR genotype (Bronstrup et al., 1998; Fohr et al., 2002). Thus, adequate folic acid supplementation could prepare the oocyte for the increased needs during preimplantation embryo genesis. Interestingly, the removal of the zona pel lucida of mouse embryos immediately following fertilization results in hypomethylation of the embryonic DNA (Ribas et al., 2006); could zona-free embryos equilibrate their methyl donors with the culture media more readily than zona-intact embryos, thus reducing their intracellular stores?

In conclusion, although we did not find any association of the MTHFR C677T and A1298C polymorphisms with ongoing pregnancy or the other outcomes examined, including spontaneous loss, further studies are warranted to examine other genes in the pathway leading to the methylation of DNA and other biologic targets. Furthermore, a follow-up study to examine birth outcomes from this cohort of patients is indicated, as more long-term developmental consequences are possible in this group of patients. Finally, this study currently suggests that MTHFR genotypes cannot explain IVF failure in women taking periconceptual folic acid.

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References


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