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Investigating the impact of the Down syndrome related common MTHFR 677C>T polymorphism in the Danish population

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Abstract. Chromosomal aneuploidy consists the leading cause of fetal death in our species. Around 50% of spontaneous abortions until 15 weeks of gestational age are chromosomally aneuploid, with trisomies accounting for 50% of the abnormal abortions. Trisomy 21 is the most common chromosome abnormality in liveborns and is usually the result of nondisjunction of chromosome 21 in meiosis in either oogenesis or spermatogenesis. To investigate the relationship between folate metabolism and Down syndrome (DS) in a Danish population, we analyzed the common 677C>T genetic polymorphism in the methylenetetrahydrofolate reductase (*MTHFR*) gene. Our cohort consisted of 181 mothers of children with DS versus 1,084 healthy controls. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to examine the *MTHFR* 677C>T polymorphism. No significant association between the polymorphism and the risk for DS was found. We conclude that the common *MTHFR* 677C>T polymorphism is not likely to be a maternal risk factor for DS in our cohort and that the difference to previous studies can probably be explained by small sample size or geographic variation in gene polymorphisms involving gene-nutritional or gene-gene or gene-nutritional-environmental factors.

Keywords: Down syndrome, nondisjunction, *MTHFR*, polymorphism, risk factor

1. Introduction

Most of our knowledge about chromosomal nondisjunction in man comes from studies in trisomy 21, the most frequent of the autosomal trisomies in liveborns. With an incidence of 1–2:1,000 in human populations [1], trisomy 21 is the most common single cause of mental retardation. The clinical entity, known as Down syndrome (DS), is usually the result of malsegregation (nondisjunction) of chromosomes 21. Advanced maternal age is the only well documented risk factor for maternal meiotic nondisjunction, but there is still a surprising lack of understanding of the cellular and molecular mechanisms underlying meiotic nondisjunction. It would be of great medical importance to identify younger mothers at increased risk for DS.

Folic acid is essential for the *de novo* synthesis of nucleotide precursors for normal DNA synthesis and is also essential for normal cellular methylation reactions. Chronic folate/methyl deficiency *in vivo* and *in vitro* has been associated with abnormal DNA methylation [2–5], DNA strand breaks [6–8], altered chromosome recombination [9,10], and aberrant chromosome segregation [11–15]. On the basis of this evidence, James et al. [16] suggested the possibility that gene-nutrient interactions associated with abnormal folate metabolism and DNA hypomethylation might increase the risk of chromosome nondisjunction.

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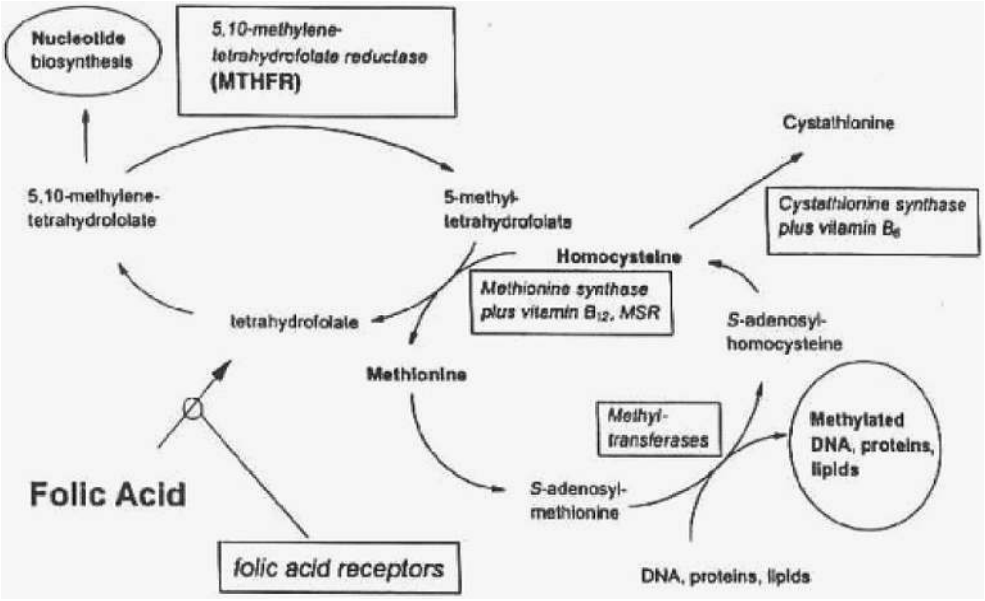

Fig. 1. Diagram illustrating folate metabolism. The *MTHFR* gene catalyzes the synthesis of 5-methyltetrahydrofolate. The reduction in enzyme activity associated with the 677C>T *MTHFR* polymorphism raises the dietary requirement for folic acid to maintain normal remethylation of homocysteine to methionine.

The *MTHFR* (methylenetetrahydrofolate reductase) gene catalyzes the synthesis of 5-methyltetrahydrofolate, the methyl donor for the B_{12}-dependent remethylation of homocysteine to methionine via the methionine synthase reaction. The enzyme is responsible for the reduction of methylenetetrahydrofolate, which is a key single-carbon donor that takes part in nucleotide synthesis; S-adenosylmethionine synthesis; remethylation of homocysteine to methionine; and the methylation of DNA, proteins, neurotransmitters, and phospholipids (Fig. 1). The reduction in enzyme activity associated with the 677C>T *MTHFR* polymorphism raises the dietary requirement for folic acid to maintain normal remethylation of homocysteine to methionine [17]. Persons with the *MTHFR* 677C>T polymorphism in homozygosity have lower serum folate and a higher homocysteine concentration than persons with the CC genotype [18]. Preliminary studies had implicated the *MTHFR* 677C>T polymorphism as a maternal risk factor for DS [16]. Lymphocytes from women consuming a controlled folate-deficient diet were found to have significantly increased frequency of kinetochore-positive micronuclei, which are surrogate markers for abnormal chromosome segregation [14]. Folate supplementation after the folate-depletion phase in the mentioned metabolic study was associated with a significant decrease in such centromeric fragments. An increased frequency of micronuclei in young mothers of DS babies and a correlation between micronuclei and the *MTHFR* 677C>T polymorphism have been shown [19, 20].

Taken together, these studies support the possibility that multifactorial gene-environment interactions that compromise maternal folate status, may promote meiotic nondisjunction and the risk of a DS conception. Hobbs et al. [21] suggested that the ability to analyze the *MTHFR* genotypes in terms of specific metabolic biomarkers – such as plasma homocysteine, folate, and/or B_{12} levels – would increase the power to detect a significant impact on DS risk. A compromise in the methionine synthase reaction caused by genetic and/or dietary factors could promote abnormal chromosome segregation by an indirect effect on oocyte DNA methylation patterns and higher-order chromatin structure. The secondary structure of pericentromeric heterochromatin, at repetitive satellite sequences, is involved in protein-DNA binding and in cohesion between sister chromatids [22–24].

Studies investigating the association between the methylenetetrahydrofolate reductase (*MTHFR*) gene 677C>T polymorphism involving folate metabolism that affects DNA methylation and synthesis with DS, have reported contradictory or inconclusive results [16,21,25–33]. We therefore analyzed the *MTHFR* 677C>T polymorphism in a population-based study of DS in Denmark, where the origin of nondis-

junction was determined by DNA microsatellite analysis in a previous study [34]. A preliminary version of the present study has been previously reported [35].

2. Materials and methods

2.1. Materials

Probands with trisomy 21 and their parents were derived from a population-based study of DS in Denmark [34]. All infants with DS were studied cytogenetically at one of the five chromosome laboratories of the country. The cases were born in the period from January 1, 1990 to March 31, 1993, and registered in the Danish Cytogenetic Central Register [36]. Throughout the period, 207 newborn infants were registered and subjected to a study of parents, chromosomes, DNA, and questionnaires including demographic characteristics. In total, 181 DNA samples of mothers of probands with non-mosaic, free trisomy 21 were available. In all cases the origin of nondisjunction was maternal meiotic (122 meiosis I errors and 55 meiosis II errors) as previously determined by DNA polymorphism analysis with microsatellites spanning the long arm of human chromosome 21 [22,34]. In four cases the origin of nondisjunction was unknown by the DNA marker analysis. Paternal and mitotic errors were excluded from the present study.

The population controls consisted of Danish newborn babies previously studied (n = 1,084) [37]. An ideal control group would have been mothers that have given birth to children without reported congenital abnormalities in the same population. However, these were not available and therefore data regarding the *MTHFR* polymorphism on newborn babies were obtained from a previous study [37]. The cases consisted of Guthrie card blood spots and were anonymously examined. The cohort investigated represented consecutive samples from all children born during a short time frame submitted to the Statens Seruminstitut, Copenhagen.

2.2. Methods

Genotyping of the *MTHFR* 677C>T polymorphism was performed with PCR amplification of genomic DNA, restriction enzyme digestion with HinfI, and agarose gel electrophoresis, using primers as described elsewhere [38].

We used the chi-square test to compare the frequency of the mutant 677T allele in the mothers (n = 177) with the frequency in population controls (n = 1,084). As different mechanisms are believed to be responsible for meiosis I and II nondisjunction, the allele frequency in mothers with a meiosis I error was compared with the frequency in mothers with a meiosis II error. Adjusted odd ratios (ORs) for DS mothers versus controls were calculated under different models as specified in Zintzaras and Lau [39].

3. Results

From the 181 maternal DNA samples, 177 were genotyped for the *MTHFR* 677C>T polymorphism, due to the fact that the PCR amplification was not successful in a number of cases. The *MTHFR* 677C>T genotype and allele distributions in parents of trisomy 21 probands and population controls are shown in Table 1. The frequency of the 677T allele in the mothers (27.7%) was not significantly different from the frequency in Danish controls (29.0%, $\chi^2 = 0.26$, $p > 0.60$, $n = 1{,}084$). Furthermore, there was no difference in the frequency of the 677T allele between mothers with meiosis I errors (27.5%) and mothers with meiosis II errors (28.2%, $\chi^2 = 0.02$, $p > 0.80$). The adjusted ORs for DS mothers versus controls were 0.86 (additive model), 0.93 (dominant model), and 1.69 (recessive model). The adjusted ORs for mothers MI versus controls were 0.93 (additive model), 0.89 (dominant model), and 0.99 (recessive model). The ORs for mothers MII were 0.67, 1.05 and 0.64 for the additive, dominant and recessive model, respectively.

4. Discussion

Advanced maternal age remains the only well documented risk factor for maternal meiotic nondisjunction, but there is, however, still a surprising lack of understanding of the basic mechanisms underlying nondisjunction and the maternal age effect. The first molecular correlate of nondisjunction in humans is altered recombination, meiosis I errors being associated with reduced recombination in both maternal and paternal meiosis [40,41] and maternal meiosis II errors with increased recombination between the nondisjoined chromosomes [42]. A two-hit model of nondisjunction has been proposed, in which the first hit is the prenatal establishment of a susceptible chiasmate configuration,

Table 1
MTHFR 677C>T genotype and allele frequencies in mothers of trisomy 21 probands and population controls

	Genotypes (%)			Alleles (%)	
	CC (%)	CT (%)	TT (%)	C (%)	T (%)
Mothers	92 (52.0)	72 (40.7)	13 (7.3)	256 (72.3)	98 (27.7)
Controls*	545 (50.3)	449 (41.4)	90 (8.3)	1539 (71.0)	629 (29.0)
Mothers MI[a]	65 (53.3)	47 (38.5)	10 (8.2)	177 (72.5)	67 (27.5)
Mothers MII[b]	27 (49.1)	25 (45.5)	3 (5.4)	79 (71.8)	31 (28.2)

*Data from Gaustadnes et al., 1999.
MI[a] = maternal meiosis I errors.
MII[b] = maternal meiosis II errors.

and the second hit is disruption of a 'meiotic process' that increases the risk of nondisjunction of the susceptible tetrad (probably the source of the maternal age effect) [42].

There is evidence that some mothers of infants with trisomy 21 have abnormal folate and methyl metabolism, resulting in DNA hypomethylation, which is associated with chromosomal instability, impaired segregation, and aneuploidy [5]. To explore the impact of the abnormal folate metabolism on Down syndrome, we investigated the association of the common *MTHFR* 677C>T polymorphism in the Danish population. Findings from the third National Health and Nutritional Examination Survey on 6,793 participants have shown a 22% reduction of serum folate and a 26% increase of homocysteine concentration in persons with the *MTHFR* 677TT genotype compared to persons with the *MTHFR* 677CC genotype [18]. Moderate daily folic acid intake significantly reduced the difference in homocysteine concentrations between those with the CC and TT genotypes [18]. A study in Denmark has shown a dietary folate of 280 μg/d in younger women, which is similar to the intake of 300 μg/d recommended in the Nordic countries [43]. In the Danish National Birth Cohort, the mean intake of folic acid, estimated on the basis of a mid-pregnancy dietary questionnaire (n = 54,344), was 352 μg/d [44]. These studies in Danish women demonstrate that Denmark is not a high folic acid intake country. A high folic acid intake could possibly mask an influence of the TT genotype, provided that there is an association between the T allele and DS. Our study failed to find a significant difference between the frequency of the 677T allele in the mothers (27.7%) and the frequency of controls (29.0%). Moreover, we found that frequencies of *MTHFR* 677C/T; either heterozygous (CT) or homozygous (TT) were similar among case mothers (40.7% and 7.3%, respectively) and controls (41.4% and 8.3%, respectively). In addition, the sum of both CT and TT variants was 48.0% for case mothers versus 49.7% for controls. Although our control group was not ideal, our results suggest that there is no association between the T allele and DS in the Danish population.

Several groups have investigated, in the mothers of affected individuals, the *MTHFR* polymorphism involved in folate metabolism. Some studies have reported increased frequency of the 677C>T *MTHFR* alleles, overall or in subgroups, but the results have not been consistent [16,21,25–34]. Studies from the USA [16, 21] indicated abnormal folate metabolism in DS mothers with a higher frequency of the *MTHFR* 677C>T allele in the case mothers compared to control mothers. However, the study by James et al. [16] was based on a small number of case mothers (n = 57) from a geographically diverse population, and the parental origin of nondisjunction was not determined. Other studies have shown an association between the MTHFR 677C>T polymorphism and DS when the polymorphism appeared to act without a multiplicative interaction [30,33,45] or combined with other polymorphisms [31,45–47]. Another study [48] reported that the *MTHFR* 677C>T polymorphism is associated with a greater risk of having a child with DS in North America, Ireland and the Netherlands, but has no influence on DS risk in France and Sicily.

Other studies failed to demonstrate an association between the *MTHFR* 677C>T polymorphism and DS in France [25,48,49], Italy [27,48,50,51], Ireland [26], Turkey [29], Brazil [32] and Northern India [52]. Coppedè et al. [50] concluded that the *MTHFR* 677C>T polymorphism is not an independent risk factor for a DS offspring at a young maternal age, however, a role for the combined genotypes between the *MTHFR* gene and the reduced folate carrier gene (*RFC-1*) in the risk of DS pregnancies among young Italian women cannot be excluded. Martin et al. [52] undertook a systematic identification of common polymorphisms in the *MTHFR* gene. They resequenced the *MTHFR* gene and performed functional genomic studies using a mammalian expression system. They concluded that

it is possible that coding SNPs in this and other folate metabolizing genes may be risk factors for DS, but this needs investigation in the Danish or other populations.

Recently, it has been suggested that there could be at least three possible explanations for the conflicting results obtained when considering the maternal diet as a possible risk factor for having a child with DS [47, 54,55]. A first explanation derives from the fact that the studies were performed in populations with various levels of folic acid intake. Moreover, several investigators measured plasma homocysteine, folate and vitamin B_{12} values during or soon after pregnancy, while others some years after conception and/or in a restricted group of subjects [16,21,47]. The second explanation comes from the observation that most of the nondisjunction events leading to DS occur at maternal meiosis I during the foetal development of the mother in the maternal grandmother body [56]. Therefore, it would be the maternal grandmother whose diet might be significant for the formation of eggs carrying two copies of chromosome 21 [47,55]. A third explanation comes from the observation that several genes participating in folate metabolism are located on chromosome 21 and therefore over-expressed in developing fetuses with DS [54, 55]. This could lead to complex interactions between the maternal diet (and genotype) and the foetal folate demand resulting from its trisomic genotype. These interactions could be relevant in selecting those embryos that will survive up to the birth [54].

At present, several important biological aspects on the homocysteine cycle are known, including a) the biochemical structure and function of the *MTHFR* enzyme, b) the biological basis for the effect of the different *MTHFR* 677C>T genotypes on homocysteine levels, c) that folate is not synthesized by the organism that obtained it from the diet, d) that TT homozygotes will be at particular risk when their folate status is low because the mutant enzyme requires much higher levels of folate than the physiological one to stabilize the binding of flavin-adenosine-dinucleotide (FAD), e) that the release of flavin is prevented by increasing the levels of folate, and, f) that the cystathionine-beta-synthase gene is located in chromosome 21. Together, these facts suggest that destabilization of the homocysteine cycle may be modified by some embryonic and maternal genotypes, as well as by maternal nutritional status and life style [54,57].

Since homocysteine levels vary as a function of several factors, including pregnancy, folate intake and combinations of genetic polymorphisms of metabolic enzymes, the conflicting results obtained so far in different populations are not surprising, but rather reflect this multifactorial nature [58]. Although there was a lack of an ideal control group in our study, it appears that the 677C>T *MTHFR* polymorphism involved in folate metabolism is not a risk factor for Down syndrome in a Danish population and that the distinct data produced in different geographical areas may be explained by differences in the nutritional environment and genetic characteristics of the populations. A development of this theory could include gene-nutritional or gene-gene or gene-nutritional-environmental factors, which need to be addressed in further studies.

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