



Association of neural tube defects in children of mothers with MTHFR 677TT genotype and abnormal carbohydrate metabolism risk: a case-control study

N.M. Cadenas-Benitez¹, F. Yanes-Sosa¹, A. Gonzalez-Meneses^{1,2},
L. Cerrillos^{1,3}, D. Acosta^{1,4}, J.M. Praena-Fernandez⁵, O. Neth⁶,
I. Gomez de Terreros^{1,2} and P. Ybot-González¹

¹Grupo de Neurodesarrollo, Unidad de Gestión de Pediatría, Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío, Centro Superior de Investigaciones Científicas, Universidad de Sevilla, Seville, Spain

²Unidad de Dismorfología y Metabolismo, Servicio de Pediatría, Hospital Infantil Virgen del Rocío, Hospital Universitario Virgen del Rocío, Seville, Spain

³Unidad de Gestión Clínica de Obstetricia, Ginecología y Patologías Mamarias, Hospital Universitario Virgen del Rocío, Seville, Spain

⁴Unidad de Gestión Clínica de Endocrinología y Nutrición, Hospital Universitario Virgen del Rocío, Seville, Spain

⁵Statistics, Methodology and Research Evaluation Unit, Andalusian Public Foundation for Health Research Management, Hospital Universitario Virgen del Rocío, Seville, Spain

⁶Unidad de Enfermedades Infecciosas e Inmunopatología Pediátrica, Hospital Infantil Virgen del Rocío, Hospital Universitario Virgen del Rocío, Seville, Spain

Corresponding author: P. Ybot-González
E-mail: pachybot@yahoo.co.uk

Genet. Mol. Res. 13 (1): 2200-2207 (2014)

Received August 29, 2013

Accepted December 9, 2013

Published March 26, 2014

DOI <http://dx.doi.org/10.4238/2014.March.26.8>

ABSTRACT. Abnormalities in maternal folate and carbohydrate

metabolism have both been shown to induce neural tube defects (NTD) in humans and animal models. However, the relationship between these two factors in the development of NTDs remains unclear. Data from mothers of children with spina bifida seen at the Unidad de Espina Bífida del Hospital Infantil Virgen del Rocío (case group) were compared to mothers of healthy children with no NTD (control group) who were randomly selected from patients seen at the outpatient ward in the same hospital. There were 25 individuals in the case group and 41 in the control group. Analysis of genotypes for the methylenetetrahydrofolate reductase (MTHFR) 677CT polymorphism in women with or without risk factors for abnormal carbohydrate metabolism revealed that mothers who were homozygous for the MTHFR 677TT polymorphism and at risk of abnormal carbohydrate metabolism were more likely to have offspring with spina bifida and high levels of homocysteine, compared to the control group. The increased incidence of NTDs in mothers homozygous for the MTHFR 677TT polymorphism and at risk of abnormal carbohydrate metabolism stresses the need for careful metabolic screening in pregnant women, and, if necessary, determination of the MTHFR 677CT genotype in those mothers at risk of developing abnormal carbohydrate metabolism.

Key words: Spina bifida; Neural tube defects; Folic acid; Diabetes; MTHFR

INTRODUCTION

Neural tube defects (NTDs) caused by incomplete neural tube closure are among the most prevalent and severe of all birth defects. NTDs affect approximately 8 to 10 per 10,000 live births in Spain and comprise a broad spectrum of phenotypes ranging from spina bifida to anencephaly (Casimiro-Soriguer et al., 2006). The multifactorial etiology of NTDs includes genetic predisposition, maternal nutritional deficiencies and other environmental factors (Eskes, 1998; Melvin et al., 2000). Notably, folate deficiency during pregnancy is one of the most important risk factors for NTDs and the periconceptional supplementation of folic acid has proven to prevent 50-70% of NTDs (Wald et al., 1991; De Wals et al., 2007).

Polymorphic variants of some of the key enzymes involved in folate metabolism have been shown to reduce its bioavailability or bioactivity. Variants of methylenetetrahydrofolate reductase (MTHFR) with a C to T substitution at nucleotide 677, for instance, have reduced activity and have been linked to the occurrence of NTDs in some populations (Eskes, 1998; Melvin et al., 2000; van der Put and Blom, 2000). Individuals with this variant usually have significantly reduced plasma folate, vitamin B12 and methionine, but increased levels of homocysteine (Eskes, 1998).

Another factor known to be associated with an increased risk of NTDs is maternal diabetes, 2- to 3-fold higher than in the non-diabetic population (Becerra et al., 1990). Interestingly, it has been reported that folic acid reduces the incidence of NTDs in offspring in a mouse model of diabetes (Oyama et al., 2009). To date, however, the interaction between maternal genetic and environmental factors in determining the risk of NTDs has not been examined. In this study, we analyzed the relationship between MTHFR polymorphisms and risk factors for abnormal carbohydrate metabolism in relation to the occurrence of spina bifida.

MATERIAL AND METHODS

Subjects

A case-control study was undertaken in mothers of children with spina bifida seen at the Unidad de Espina Bífida del Hospital Infantil Virgen del Rocío (case group) and mothers of healthy children with no NTD (control group) who were randomly selected from patients seen in the outpatient ward in the same hospital. The mothers were aged 20 to 43 years in the case group and 23 to 42 years in the control group. The local ethics committee approved the protocol and written informed consent was obtained from the participants and their relatives. Cases and controls with a history of folic acid and vitamin consumption in the previous 3 months or chronic diseases related to the study and non-Caucasian families were excluded from the analysis.

Homocysteine determination

EDTA blood samples were collected and homocysteine levels were determined with the IMX System (Abbott Division Diagnostics, Oslo, Norway) using the fluorescence polarization immunoassay (FPIA). Plasma homocysteine $\geq 15 \mu\text{M}$ was considered to be indicative of hyperhomocysteinemia.

Genetic analysis

DNA was extracted using the QIAamp DNA Blood Midi kit (QIAGEN GMBH Hilden Germany) and stored at -20°C . Polymorphisms for MTHFR C677T were analyzed by RFLP-PCR as previously described (van der Put et al., 1995).

Clinical data collection

The following sources were used: clinical histories and databases (SIDCA, Weblab 3.3 and Weblab historic) of the Hospital Virgen del Rocío, databases from the Hospital de Valme (Omega 3000), Asociación Sevilla de Padres con Hijos con Espina Bífida e Hidrocefalia (ASPHEBH), telephone interviews and review of pregnancy, and birth cards.

The O'Sullivan test was used as an indication of risk of developing gestational diabetes and considered positive in pregnant women with glucose values $\geq 130 \text{ mg/dL}$ (Metzger and Coustan, 1998). Overweight was defined as body mass index (BMI) between $25\text{-}29.9 \text{ kg/m}^2$ and obesity as $\text{BMI} \geq 30 \text{ kg/m}^2$.

Statistical analysis

Statistical calculations were performed using IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp. Data are reported as frequency distributions for categorical variables and as median and interquartile range for continuous variables (not normally distributed) or mean and range. We used the Mann-Whitney U-test to detect changes within groups in continuous variables and the Pearson χ^2 test or Fisher exact

test for categorical variables. For the groups, we obtained the odds ratio (OR) and 95% confidence interval (CI). Statistical significance was set at $P < 0.05$.

RESULTS

Evaluation of polymorphism distribution

There were no significant differences in the frequency of MTHFR C677T genotypes between case and control subjects (Table 1). However, the frequency of T in the case group was significantly higher than in the control group ($P = 0.025$, OR = 2.28, 95%CI = 1.102-4.716).

Table 1. Incidence of spina bifida in the study groups.

	Controls (N = 41)	Cases (N = 25)	OR (95%CI)	P
Mothers MTHFR 677TT ^a	5 (13%)	7 (29.2%)	2.80 (0.78-10.06)	0.115
Mothers + risk factor ^b	22 (53.7%)	16 (64%)	1.53 (0.55-4.26)	0.411
Mothers MTHFR 677TT + risk factor ^c	2 (4.9%)	6 (24%)	6.16 (1.13-33.43)	0.046*

^aRecessive inheritance: Selected group: Mothers homozygous for MTHFR C677T, MTHFR677TT. Unselected group: Mothers wild type and heterozygous for MTHFR C677T. ^bMothers + risk factor: Selected group: Mothers with risk factors for abnormal carbohydrate metabolism. Unselected group: Mothers with no risk factors for abnormal carbohydrate. ^cMothers MTHFR 677TT + risk factor: Selected group: Mothers with risk factors for abnormal carbohydrate metabolism and homozygous for MTHFR C677T, MTHFR 677TT. Unselected group: Mothers with risk factors for abnormal carbohydrate metabolism and wild type and heterozygous for MTHFR C677T; Mothers with no risk factors for abnormal carbohydrate metabolism and homozygous for MTHFR C677T; mothers with no risk factors for abnormal carbohydrate metabolism and wild type and heterozygous for MTHFR C677T. *Fisher's exact test.

Evaluation of the variables analyzed in relation to carbohydrate metabolism

Despite the lack of a statistically significant difference, cases were more often overweight or obese when they conceived (36% of cases vs 22% of controls). Unfortunately, O'Sullivan test results were only available for 10 individuals from the case group. The median glucose level in that group was 120.5 mg/dL (range = 99-139) compared to a median of 116.5 mg/dL (range = 100.5-155.5) in the 16 individuals from the control group.

We considered that mothers at risk of developing abnormal carbohydrate metabolism were those that showed at least one of the following risk factors: personal history of diabetes mellitus or gestational diabetes, diabetes mellitus in a first-degree family member, BMI ≥ 25 kg/m² in the initial stages of pregnancy, or O'Sullivan test ≥ 130 mg/dL. According to this classification, mothers classified as at risk of abnormal carbohydrate metabolism were more likely to have a child with spina bifida (64% in the at-risk group vs 53.7% in mothers without risk factors; Fisher exact, $P = 0.411$, Table 1).

Evaluation of risk factor combinations: Relationship between maternal C677T polymorphism and abnormal carbohydrate metabolism

Given the higher incidence of T in the case group, we decided to analyze the data on the basis of two models, either dominant, in which the effect of both homozygotes and heterozygotes are equal, or recessive, in which effects are expected only from homozygotes. Analysis

of genotypes for the MTHFR 677CT polymorphism in women with or without risk factors for carbohydrate metabolism revealed that mothers who were homozygous for the MTHFR 677TT polymorphism and at risk of abnormal carbohydrate metabolism were more likely to have offspring with spina bifida (24% of mothers in the case group vs 4.9% in control group; Table 1).

Homocysteine

Evaluation of homocysteine concentration

Since elevated levels of homocysteine have been proposed as a risk factor for NTDs (Stegers-Theunissen et al., 1994), we evaluated homocysteine levels in the case and control groups. However, no difference was observed between the groups (Table 2).

Table 2. Levels of homocystein in the groups under study.

		Hcy (μ M)	P
	MC (N = 41)	8.9 (7.3-10.9)	0.182 π
	MP (N = 25)	9.52 (7.46-13.3)	
Mothers MTHFR 677TT	MC hom (N = 5)	8.47 (7.08-10.05)	0.062 π
	MP hom (N = 7)	18.49 (11.57-22.51)	
Mothers + risk factor	MC (N = 22)	8.74 (6.69-11.79)	0.156 π
	MP (N = 16)	12.07 (7.34-18.92)	
Mothers MTHFR 677TT + risk factor	MC hom+risk factor + (N = 2)	10.66 (9.65-11.67)*	
	MP hom+risk factor + (N = 6)	18.78 (9.52-48.03)*	

Hcy = homocysteine; Hom = homozygous for MTHFR, 677 TT; Het = heterozygous for MTHFR, 677 CT; wt = wild type for MTHFR, 677 CC; MC = mothers of control group; MP = mothers of case group; risk factor + = presence of risk factors for abnormal carbohydrate metabolism. Homocysteine levels are represented as median and interquartile range apart from the data marked (*), which represents the range. π = Mann-Whitney test.

Maternal C677T polymorphism and homocysteine concentration

Since homocysteine levels are likely to depend on maternal genetic influence (Kluijtmans et al., 1997), we assessed homocysteine levels in both the maternal MTHFR dominant and recessive inheritance genotypes for determination of NTD risk. While we did not detect in the dominant inheritance analysis any difference in the homocysteine levels between the genotypes, in the analysis of recessive inheritance, those mothers in the case group (MP) with a MTHFR 677TT genotype had higher levels of homocysteine than those in the control group (MC; P = 0.062; Table 2).

Homocysteine levels in mothers at risk for abnormal carbohydrate metabolism

Given the link between elevated plasma homocysteine levels and the risk of developing diabetes or its complications (Hoffman, 2011), we compared levels of homocysteine in subjects with and without risk factors for abnormal carbohydrate metabolism. No significant difference was detected between the groups, although there was a trend towards higher homocysteine levels in mothers from the case group who also had risk factors for abnormal car-

bohydrate metabolism (12.07 vs 8.74 μM ; Table 2). In addition, mothers from the case group with risk factors for abnormal carbohydrate metabolism and with MTHFR 677TT genotype had the highest levels of homocysteine (Table 2, homocysteine $\geq 15 \mu\text{M}$, which can be considered indicative of hyperhomocysteinemia).

DISCUSSION

In this study we observed an increased risk of NTDs in the offspring of mothers with an MTHFR 677TT genotype along with risk factors for abnormal carbohydrate metabolism. In addition, homocysteine levels were found to be higher in mothers of children with spina bifida who were carriers of MTHFR 677TT and also at risk for abnormal carbohydrate metabolism. These results further support a correlation between genetic and environmental risk factors in the development of NTDs.

Risk of developing abnormal carbohydrate metabolism

It is well established that maternal diabetes during pregnancy is associated with a high incidence of congenital malformations, including NTD (Loeken, 2006). In agreement with this, our study showed that women at risk of developing abnormal carbohydrate metabolism have a higher risk of having a child with an NTD. Interestingly, this risk was greatly increased if the mother is also a carrier of the MTHFR 677TT mutation.

Homocysteine

The 677C->T mutation in the MTHFR gene causes an alanine to valine substitution in the protein, affecting the activity of this enzyme in both homozygous and heterozygous subjects and resulting in an accumulation of homocysteine (van der Put et al., 1995; Eskes, 1998). In our study, significantly higher levels of homocysteine were detected in mothers who were carriers of MTHFR 677TT, consistent with the results of previous studies (Eskes, 1998). The risk of developing abnormal carbohydrate metabolism was not associated with differences in the level of homocysteine. However, higher levels of homocysteine were observed in those mothers with the MTHFR TT genotype and also at risk of developing abnormal carbohydrate metabolism.

A potential mechanism to explain the increased frequency of NTDs associated with maternal diabetes is oxidative stress. Increased oxidative stress is widely accepted to be involved in the development and progression of diabetes and its complications (Maritim et al., 2003). Moreover, analysis of gene expression in mouse embryos with NTDs from a model of maternal diabetes revealed that there was an enrichment of genes containing binding sites for transcription factors involved in the response to oxidative stress (Pavlinkova et al., 2009). It is also well documented that oxidative stress inhibits the expression of *Pax-3*, which is essential for neural tube closure during embryogenesis (Chang et al., 2003). Given that high levels of homocysteine also induce oxidative stress (Heydrick et al., 2004; Albu et al., 2012), women with moderate levels of blood glucose ($\geq 120 \text{ mg/dL}$) who also have an MTHFR TT genotype may have similar levels of oxidative stress as those of a fully diabetic woman. Further studies should therefore assess whether changes in oxidative stress explain the observed interaction between MTHFR genotype and abnormal carbohydrate metabolism.

In our analysis, we considered, as suggested by the 4th Workshop-Conference on Gestational Diabetes Mellitus (Metzger and Coustan, 1998), glucose levels ≥ 130 mg/dL as a positive O'Sullivan test. However, in Spain, the diabetes and gestation guide (GEDE, 2005) still considers ≥ 140 mg/dL as pathological levels. Using a cutoff of 140 mg/dl, 79% of women with gestational diabetes would be detected (87% specificity) compared to 100% of women (78% specificity) using the 130 mg/dL cutoff (ADA, 2001; Carrera, 2005). Our results therefore stress the importance of careful metabolic screening in pregnant women, and, if necessary, analysis of MTHFR 677CT genotype in those mothers at risk of developing abnormal carbohydrate metabolism. Nevertheless, the results of this study are limited by the small number of subjects, and a larger sample will be required to confirm and extend our findings.

ACKNOWLEDGMENTS

Research supported by Instituto de Salud Carlos III, project #CP08/00111 and #PS09/00050 (to P. Ybot-González) and Consejería de Salud de la Junta de Andalucía, project #PI-0438-2010 (to P. Ybot-González) and #PI-0045-2001 (to I. Gómez de Terreros). The authors gratefully acknowledge Phil Stanier and Iain Patten for valuable comments during the writing of the manuscript and all the families who took part in this study.

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