A dihydrofolate reductase 2 (DHFR2) variant is associated with risk of neural tube defects in an Irish cohort but not in a United Kingdom cohort

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To the Editor,
Folate-responsive neural tube defects (NTDs) are a group of congenital malformations that can be prevented by the periconceptional consumption of the B-vitamin, folic acid (Berry et al., 1999; Kirke, Daly, & Elwood, 1992; Laurence, James, Miller, Tennant, & Campbell, 1981). NTD occurrence rates vary among populations with a range of 6–60 per 10,000 pregnancies worldwide (Molloy, Pangilinan, & Brody, 2017). Because NTDs are relatively common, and can be severe or fatal defects, preventing folate-responsive defects is a major public health priority (Bailey, West Jr., & Black, 2015). Voluntary or mandatory folic acid fortification programs have been implemented across the globe, with variable success (Khoshnood et al., 2015). Given such wide public health implications, there is a need to further understand the mechanism by which folate-responsive NTDs occur. Although NTDs are known to have both nutritional and genetic risk factors (Bailey et al., 2015), much of the genetic component remains to be discovered. An unbiased genome-wide association study is an appealing approach but has not yet been performed due to the difficulty of obtaining a sufficient number of affected participants. In contrast, candidate genes studies are feasible and have some potential advantages compared with a GWAS. First, because variant selection can use linkage disequilibrium to inform the selection of variants, a tailored approach can provide more complete variant coverage of the gene(s) in question. Second, querying this targeted search space may identify small but real association signals that would be lost in the multiple-test correction of a full-scale GWAS.

Genetic investigations have focused on genes that either metabolize or transport folate due to the well-established protective effect of preconceptional use of folic acid supplements (Czeizel & Dudas, 1992; MRC Vitamin Study Research Group, 1991). Many genetic association studies have considered variants within folate pathway genes as candidate risk factors in the search of genetic variants that may increase the population and an individual’s risk of an NTD (Molloy et al., 2017). We previously reported an association study of 82 candidate genes in our Irish NTD cohort (Pangilinan et al., 2012), as well as a replication study (Pangilinan et al., 2014). While associations in MTHFD1 (Brody et al., 2002; Jiang, Zhang, Wei, Sun, & Liu, 2014; Meng, Han, & Zhuang, 2015) and MTRR (Ouyang, Li, Liu, Chang, & Wu, 2013; Yadav, Kumar, Yadav, Mishra, & Rai, 2015) were among the most significant findings from these and other studies, the MTHFR 677C>T (rs1801133) variant remains the most consistently associated genetic modifier of NTD risk (Botto & Yang, 2000; van der Put et al., 1995; Yadav et al., 2015; Yan et al., 2012; Yang, Chen, Wang, Ding, & Liu, 2015; Zhang et al., 2013). This variant is associated with biomarkers of folate status, including changes in levels of serum folate, red cell folate and homocysteine (Shane et al., 2018).

We report here our evaluation of the folate retrogene known as dihydrofolate folate reductase 2 (DHFR2, formerly DHFRL1) (McEntee...
et al., 2011) as a candidate gene for NTD risk. We previously reported that a 19 bp intronic allele of dihydrofolate reductase (DHFR) may decrease risk of NTDs (Parle-McDermott et al., 2007). DHFR mediates the entry of dietary folic acid into folate metabolism. Owing to this key role, DHFR has been extensively studied as a drug target (methotrexate) and a selection tool in cell culture (McEntee et al., 2011). In addition to DHFR, humans and other primates have acquired an a second gene family member, making DHFR2 a compelling candidate for harboring variation that might alter NTD risk. Moreover, as a relatively newly identified gene, DHFR2 has not been considered as a candidate in many human disease contexts.

Our initial cohort in the investigation of DHFR2 consisted of 595 trio families that included an affected case and one or both parents plus a control sample of 1,000 individuals. This cohort has been described previously (Brody et al., 2002; Pangilinan et al., 2014; Shields et al., 1999). In brief, NTD cases and their parents were recruited throughout Ireland (1993–2004), and controls were randomly selected from women attending their first prenatal visit in Dublin (1986–1990). We successfully genotyped seven single nucleotide polymorphisms (SNPs) across the DHFR2 gene region by detection of allele-specific primer extension using matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, California). The DHFR2 gene harbors only two common (MAF > 0.05) SNPs in the European population (EUR in 1000G, [Genomes Project Consortium et al., 2015]); these and five SNPs from the 10 kb flanking regions were selected to ensure sufficient coverage of the gene and its proximate cis-regulatory elements. The SNPs spanned the genomic region which contained the open reading frame (ORF) and extended 4.8 kb and 4.2 kb upstream and downstream of the ORF respectively. The average SNP call rate across the sample groups ranged from 95.1 to 97%. The average duplicate concordance rate was 98.2%. Association analysis for each SNP included the transmission disequilibrium test (TDT) using PLINK in Haploview version 1.0 and testing for homozygous genotype effect mapped) for DHFR2 and genotypes for SNP rs17855824 were down-loaded from the Geuvadis RNA Sequencing project (Lappalainen et al., 2013). Of the 462 individuals with RPKM values for the DHFR2 gene, 381 had CC genotypes, 77 CT, and 4 TT. An ANOVA for relative mRNA expression between the rs17855824 genotypes was not significant ($F_{2,459} = 0.21, p = .05$). Samtools’ mpileup was used with the raw BAM files for the heterozygous individuals to find the number of

| DHFR2 SNP #a | Allele OTb | T:U ratio c | $\chi^2$ | p-value | MAF |
|-------------|-----------|--------------|--------|--------|-----|
| rs7645522   | A         | 107:71       | 7.281  | .007   | G (0.13) |
| rs11927165  | T         | 24:19        | 0.581  | .4458  | C (0.03) |
| rs10454213  | A         | 105:66       | 8.895  | .0029  | G (0.13) |
| rs17855824  | C         | 97:62        | 7.704  | .0055  | T (0.13) |
| rs61739170  | C         | 130:125      | 0.098  | .7542  | C (0.24) |
| rs7644176   | G         | 25:18        | 1.14   | .2858  | T (0.03) |
| rs7653521   | C         | 177:158      | 1.078  | .2992  | C (0.43) |

| TABLE 1 Transmission disequilibrium test analysis of DHFR2 SNPs in an Irish NTD cohort |

aSNPs in bold share high LD ($D' = 1; r^2 ≥ .995$).
bAllele that was over-transmitted.
cTransmitted:untransmitted ratio.
reads with T and C alleles, a Pearson’s chi-square test did not find a significant difference in allele abundance. We then asked whether \textit{DHFR2} \textit{rs17855824} might act as an eQTL in specific tissues. Although there are \textit{DHFR2} eQTLs in and around the gene, the Genome-Tissue Expression project (GTEx V8) shows that \textit{rs17855824} is an eQTL for \textit{PROS1} but not \textit{DHFR2} in cultured cells and tibial artery (https://www.gtexportal.org/home/, [GTEx Consortium, 2013]).

We next used a second UK cohort to assess whether a similar significant TDT association would be replicated. This cohort has been previously described (Pangilinan et al., 2010). Briefly, 497 NTD cases and their parents, when available, were recruited throughout the UK (2001–2003) with the assistance of the UK Association for Spina Bifida and Hydrocephalus. We genotyped two \textit{(rs10454213 and rs7645522)} of the three Irish cohort associated SNPs. Genotyping was performed by LGC KBiosciences (The United Kingdom) and included at least 67 duplicate samples per SNP. In addition, at least 231 samples per SNP were repeat genotyped in-house using the Sequenom platform as described above. The concordance rate for duplicate and repeat genotyped samples was 100 and 99.6%, respectively. Our TDT analysis of the UK cohort (354 complete trios) did not show a significant over-transmission of the “A” allele as observed in the Irish cohort (Table 2). In fact, a nonsignificant over-transmission of the minor allele, that is, the “G” was observed for both SNPs in the UK cohort. In the absence of a significant difference in allele transmission in the UK cohort, we conclude that this SNP does not contribute to NTD risk in this UK cohort, or the cohort was underpowered (354 complete UK trios tested compared with 440 complete Irish trios) to detect a significant difference in allele transmission.

In summary, our consideration of the human \textit{DHFR2} gene as a candidate genetic risk factor for NTD revealed mixed results. We observed an association signal in an Irish cohort but failed to replicate this in a separate, smaller UK cohort. We also assessed \textit{DHFR2} as a potential QTL for biomarkers of folate one carbon metabolism and as an eQTL. Upon correction for multiple tests, these analyses did not show any significant correlations with serum folate, red cell folate, plasma total homocysteine, plasma formate, or tissue mRNA expression levels. This is in contrast to the \textit{MTHFR} 677C>T variant which was previously found to be the major genetic modifier of these biomarkers in the same cohort (Brosnan et al., 2018; Shane et al., 2018).

We conclude that \textit{DHFR2} does not contribute to NTD risk by acting

![FIGURE 1](image_url)  
**FIGURE 1**  
Pairwise measures of linkage disequilibrium (LD) for the seven SNPs genotyped in the Irish cohort. Left: \(D^'\) measures of LD; right: \(r^2\) measures of LD. Solid blocks indicate a value of \(D^' = 1\) (red or blue) or \(r^2 = 1\) (black). Numbers in remaining blocks indicate the pairwise estimate of LD multiplied by 100 for improved legibility. Only the SNPs numbered 4 (rs17855824) and 5 (rs61739170) fall within the \textit{DHFR2} gene

**TABLE 2**  
Replication TDT analysis of \textit{DHFR2} SNPs in a UK NTD cohort

| \textit{DHFR2} SNP # | Allele OT\(^a\) | T:U ratio\(^b\) | \(\chi^2\) | \(p\)-value | MAF |
|---------------------|----------------|----------------|----------|-----------|-----|
| rs7645522           | G              | 46:31          | 2.922    | 0.0874    | G (0.13) |
| rs10454213          | G              | 46:32          | 2.513    | 0.1129    | C (0.03) |

\(^a\)Allele that was over-transmitted.  
\(^b\)Transmitted:untransmitted ratio.
as a QTL that influences folate biomarkers. The lack of an association in the UK cohort; however, does not completely rule out this gene from playing a role in NTD etiology. The accepted NTD risk factor MTHFR 677C>T variant has not shown an association in all cohorts tested (Amorim, Lima, Castilla, & Orioli, 2007; Botto & Yang, 2000) including the same UK cohort reported here (Pangilinan et al., 2014). Research is currently ongoing to elucidate the functional role of DHFR2 and its confirmation as a genetic modifier of NTD risk requires replication in another cohort. While periconceptional folic acid supplementation remains the most effective action clinicians can recommend to reduce the risk of NTDs, further research is crucial for understanding the genetic contribution to this birth defect. Candidate gene studies based on prioritizing genetic variants that may influence the folate one carbon metabolic pathway will continue to be important. Ultimately a GWAS will be required to determine in an unbiased way which genetic variants not yet considered contribute to the development of NTDs.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Anne Parle-McDermott, Lawrence C. Brody, James L. Mills, Barry Shane, Anne M. Molloy: conceived and designed the study. Faith Pangilinan and Hattice O. Abaan: performed genotyping quality control. Faith Pangilinan, Emma K. Finlay, Anne Parle-McDermott, Lawrence C. Brody: analyzed and interpreted the data. Faith Pangilinan, Anne Parle-McDermott: wrote the first draft of the manuscript. All authors reviewed, edited and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. All genotype data is available in the Supporting Information Data File.

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