Research Article

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Folate Pathway Gene Polymorphisms and Risk of Childhood Brain Tumors: Results from an Australian Case–Control Study

Kathryn R. Greenop[1], Rodney J. Scott[2,3,4], John Attia[2,5], Carol Bower[1], Nicholas H. de Klerk[1], Murray D. Norris[6], Michelle Haber[6], Sarra E. Jamieson[1], Frank M. van Bockxmeer[7,8], Nicholas G. Gottardo[1,9,10], Lesley J. Ashton[11,12], Bruce K. Armstrong[13,14], and Elizabeth Milne[1]

Abstract

Background: Recent research suggests that maternal folic acid supplementation is associated with a reduced risk of childhood brain tumors (CBT); polymorphisms in folate pathway genes could modify this association or directly influence CBT risk.

Methods: Associations between risk of CBT and folate pathway polymorphisms were investigated in a population-based case–control study in Australia (2005–2010). Cases were recruited through all Australian pediatric oncology centers and controls by national random digit dialing. Data were available from 321 cases and 552 controls. Six polymorphisms were genotyped in children and parents (*MTHFR* 677C>T, *MTHFR* 1298A>C, *MTRR* 66A>G, *MTR* 2756A>G, *MTR* 5049C>A, and *CBS* 2199 T>C). Maternal folic acid use was ascertained via questionnaire. ORs were estimated using unconditional logistic regression. Case–parent trio analyses were also undertaken.

Results: There was weak evidence of a reduced risk of CBT for the *MTRR* 66GG genotype in the child or father: ORs 0.71 [95% confidence interval (CI), 0.48–1.07]; 0.54 (95% CI, 0.34–0.87), respectively. Maternal prepregnancy folic acid supplementation showed a stronger negative association with CBT risk where the child, mother, or father had the *MTRR* 66GG genotype ($P_{\text{interaction}} = 0.07$, 0.10, and 0.18, respectively).

Conclusions: Evidence for an association between folate pathway genotypes and CBT is limited in this study. There was possible protection by the *MTRR* 66GG genotype, particularly when combined with maternal prepregnancy folic acid supplementation; these results are novel and require replication.

Impact: The possible interaction between folic acid supplementation and *MTRR* 66A>G, if confirmed, would strengthen evidence for prepregnancy folate protection against CBT. *Cancer Epidemiol Biomarkers Prev; 24(6); 931–7. ©2015 AACR.*

Introduction

Childhood brain tumors (CBT) are the second most common types of childhood cancer and have the highest mortality rate yet very little is known about their etiology. We previously reported from our case–control study, "Australian Study of Childhood Brain Tumours" (Aus-CBT) that folic acid supplementation of the mother in the month before conception is associated with a reduced risk of CBT [OR, 0.65; 95% confidence interval (CI), 0.43–0.96; ref. 1]. Given the key role that folate plays in DNA synthesis and methylation, such an association is biologically possible. Disruption to either of these mechanisms could result in DNA damage or altered gene expression, increasing the risk of cancer in the offspring if it occurs during critical periods in development (2). Polymorphisms in folate pathway genes have previously been implicated in the risk of some childhood cancers, particularly leukemia (3–5); these genes have included methyltetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), and cystathionine beta-synthase (*CBS*). Associations between folate gene polymorphisms that alter enzyme activity and risk of childhood cancer would lend support to the growing evidence that folate and relevant pathways are involved in disease development.

Two previous studies have investigated folate pathway polymorphisms in relation to CBT risk (6, 7). A Thai case–control study investigated five folate pathway polymorphisms in 73 case and 205 control children and reported an increased risk of

[1]Telethon Kids Institute, University of Western Australia, Perth, Western Australia, Australia. [2]Hunter Medical Research Institute, John Hunter Hospital, New South Wales, Australia. [3]School of Biomedical Sciences and Pharmacy, Faculty of Health, University of Newcastle, Newcastle, New South Wales, Australia. [4]Hunter Area Pathology Service, HNEHealth, Newcastle, New South Wales, Australia. [5]School of Medicine and Public Health, Faculty of Health and Medicine, University of Newcastle, Newcastle, New South Wales, Australia. [6]Children's Cancer Institute Australia for Medical Research, Lowy Cancer Research Centre, University of New South Wales, Sydney, New South Wales, Australia. [7]School of Surgery, University of Western Australia, Perth, Western Australia, Australia. [8]Department of Clinical Biochemistry, Royal Perth Hospital, Perth, Western Australia, Australia. [9]School of Paediatrics and Child Health, University of Western Australia, Perth, Western Australia, Australia. [10]Department of Paediatric Oncology/Haematology, Princess Margaret Hospital for Children, Perth, Western Australia, Australia. [11]Research Portfolio, University of Sydney, Sydney, Australia. [12]School of Women's and Children's Health, Faculty of Medicine, University of New South Wales, Sydney, Australia. [13]Sax Institute, Haymarket, New South Wales, Australia. [14]Sydney School of Public Health, University of Sydney, New South Wales, Australia.

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Corresponding Author: Elizabeth Milne, Telethon Kids Institute, PO Box 855, West Perth, Western Australia 6872, Australia. Phone: 618-9489-7756; Fax: 618-9489-7700; E-mail: Liz.Milne@telethonkids.org.au

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embryonal tumors ($n = 28$) associated with the *MTHFR* 1298CC genotype (6). This study also reported elevated associations between the *MTHFR* 677CT/TT and *MTR* 2756AG genotype and risk of glial tumors ($n = 31$); however, this association was not statistically significant. A case–control study conducted in Russia which included 284 children with high-grade glial and embryonic brain tumors and 464 adult controls observed a small reduction in CBT risk among boys ($n = 274$) associated with the *MTHFR* 677CT or TT genotype (7).

As many CBTs are diagnosed in early childhood, and may therefore have been initiated *in utero* or during germ cell synthesis, parental genotype may also influence disease risk. Potential mechanisms include DNA damage to sperm or ova, disruption to genomic integrity in the embryo, and disturbances in the normal development of the fetus. Furthermore, if relationships exist between folate pathway genotypes of child or parents and risk of CBT, it is plausible that such associations could interact with maternal folate status. Folate pathway polymorphisms in the mother or child have the potential to enhance or disrupt folate availability and could modify the influence of supplementation. Interactions between maternal folic acid use and folate genotypes have been previously reported for childhood leukemia (3, 4) and colorectal cancer in adults (8, 9).

To our knowledge, this is the first study to have investigated associations between parental folate pathway genotypes and risk of CBT. Here, we report results of an analysis of these associations in the Aus-CBT study. We also undertook subgroup analysis for genotype associations by sex or tumor subtype, and investigated whether the inverse association previously described between maternal folic acid use and CBT risk was modified by folate pathway genotypes.

Materials and Methods

Aus-CBT was a population-based case–control study that aimed to investigate nutritional, environmental, and genetic risk factors for CBT. Incident cases were identified through all 10 pediatric oncology centers in Australia; eligibility criteria were: diagnosis between 2005 and 2010, resident in Australia, and had an English speaking biological parent available. Controls were recruited by national random digit dialing (RDD) and were frequency matched to cases by age, sex, and State of residence in a ratio of approximately 3:1. Because of budgetary constraints, not all controls were asked for DNA samples. Controls matched to CBT cases diagnosed in 2005 and 2006 were originally matched to cases in our national study of childhood leukemia (Aus-ALL) that used identical recruitment methods (10). Aus-CBT and Aus-ALL were approved by the Human Research Ethics Committees at all participating hospitals.

Mailed questionnaires were used to obtain demographic characteristics and information about relevant exposures, including whether mothers took "folate supplements" (yes or no) in the month before and/or during the index pregnancy.

Blood samples for DNA analysis were collected from case children and parents during a routine hospital visit in 4 mL EDTA tubes, and refrigerated at 4°C for a maximum of 7 days before DNA extraction using the Wizard Genomic DNA Purification Kit (Promega, cat # 1620). Case children also provided a saliva sample using an Oragene kit (DNA Genotek Inc.). Approximately half the control children and parents provided saliva samples using Oragene kits, while the other half (recruited 2005–2006) provided buccal DNA using Whatman indicating FTA cards (cat # WB120211) as previously described (11). Oragene saliva samples (maximum 4 mL) were stored at room temperature before manual purification of DNA according to manufacturer's instructions (DNA Genotek, cat #OG-250).

DNA samples were collected from one or two controls per case.

Genotyping

Case and control children and their parents were genotyped for six SNPs in folate pathway genes: *MTHFR* 677C>T (rs1801133), *MTHFR* 1298A>C (rs1801131), *MTRR* 66A>G (rs1801394), *CBS* 2199T>C (rs706208), *MTR* 2756A>G (rs1805087), *MTR* 5049C>A (rs2853523). These were selected on the basis that they are associated with functional differences in the activity of their respective proteins. Genotyping was conducted by the Australian Genome Research Facility Ltd., University of Queensland (AGRF; www.agrf.org.au) using iPLEX GOLD chemistry with Sequenom MassARRAY on an Autoflex spectrometer. AGRF was blinded to case/control status, and to DNA source (blood, saliva, or buccal cells).

For quality assurance purposes, the blinded analysis at AGRF was reperformed on a randomly selected 10% of samples. Between-laboratory concordance was assessed (blinded) for *MTHFR* 677C>T at the Hunter Area Pathology Service, using methods previously described (12). Concordance between case children's genotypes derived using blood and saliva and blood and buccal samples was also examined.

Statistical analysis. Mendelian inconsistencies were identified in family data using the PedCheck software (Pedcheck 1.1, Jeff O'Connell 1997, 1999 University of Pittsburgh, Pittsburgh, PA; ref. 13; 36 inconsistencies were detected and excluded from analysis). Hardy–Weinberg Equilibrium (HWE) analysis was then performed in STATA v10 using unaffected, unrelated individuals (parents); no SNPs were found to significantly deviate from HWE (Supplementary Table S1). Quality assurance analyses showed good results: genotype call rate was 99.5%, within-lab concordance was 99.5%, between-lab concordance was 98.2%, and blood-saliva/buccal sample concordance among cases was 99.1% for Oragene samples and 96.3% for FTA card samples.

Main effects of genotype were analyzed using unconditional logistic regression in SPSS version 22 (IBM SPSS for Windows, Version 22.0, IBM Corp., 2013), adjusting for study matching variables (child's age, sex, and State of residence). To ensure comparability of the genotype models with our models on the effect of folic acid supplementation stratified by genotype, we included in all case–control analyses variables found to be potential confounders of folic acid supplementation and CBT risk in our previous report (1): maternal age, maternal education, child's year of birth, and child's ethnicity. Tumor subtype analyses were conducted using models as above, limiting cases to specific subtypes compared with all controls. Maternal folic acid supplementation in the month before pregnancy was stratified by genotype; this period of use was most associated with reduced CBT risk in our previous work (1). Evidence for an interaction was assessed formally by fitting a product term to a model (Wald test *P* values of interaction terms are reported in tables). Case–parent trio analyses were undertaken on the 246 families for whom all three genotypes were available using conditional logistic regression in StataIC version 11 (StataCorp); genotype-based ORs were estimated. This method uses the parents' genotypes to create up to

three pseudocontrols for each case child using the remaining parental genotype combinations. Each pseudocontrol is therefore matched on all environmental variables (14). Data were prepared using "pseudocc" and "gtab" commands.

Results

Detailed recruitment and participation rates have been published previously (1). Briefly, we were notified of 730 eligible CBT cases diagnosed from 2005 to 2010, of whom 568 (77.8%) were invited to participate by their treating clinician. Parents of 374 cases consented (65.8% of invited, 51.2% of eligible). Of these, 321 (86%) case children, 318 (85%) case mothers, and 261 (70%) case fathers had both genotyping and folic acid supplement data available.

Between 2005 and 2010, 3,624 families of eligible control children were identified by RDD, of whom 2,255 (62.2%) agreed to participate. In accordance with our frequency-matching quotas, 1,467 of these families were recruited to the study, 1,171 of these had DNA samples requested. Of these, 552 (47%) control children, 558 (48%) control mothers, and 483 (41%) control fathers had both genotyping and folic acid supplement data available. Figure 1 shows a flowchart of data and DNA acquisition.

Table 1 shows the demographic characteristics of cases and controls used in the current analysis. While variables used for frequency matching were similar, there were some differences in that control children were more likely to be of European ethnicity and control mothers were more likely to be older and have higher educational attainment.

Overall, few associations were seen between CBT risk and the folate pathway SNPs investigated, except for weak evidence of an inverse association for the *MTRR* 66GG genotype in the child (and trio analysis), and a slightly stronger inverse association for this genotype in the father (Table 2). There was also weak evidence of a reduced risk of CBT associated with maternal carriage of one or more mutant alleles at *MTHFR* 1298A>C or *MTR* 2756A>G. For the most part, the results of the case–parent trio analyses were consistent with those of the case–control analysis of the child's genotype; notably, both were consistent with an increased risk of CBT associated with child's carriage of one or more mutant alleles at *CBS* 2199T>C (Table 2). The results of the case–control analysis were similar when restricted to the 246 children belonging to case–parent trios; when only matching variables were adjusted for in the models, or when the analysis was restricted to families of European descent (Supplementary Tables S2–S4, respectively). As FTA samples showed slightly worse blood–buccal concordance in our quality control sample compared with Oragene samples, we repeated the analyses shown in Table 2 excluding FTA samples; the results were not materially different from those presented (Supplementary Table S5).

Combinations of *MTHFR* genotypes in the child were also examined, with wild-type genotypes for both SNPs being the reference. Where the child was heterozygous for both SNPs and had inherited mutant alleles from each parent (in trans) the OR was 0.84 (95% CI, 0.46–1.53); where only one mutant allele was present from either SNP the OR was 0.98 (95% CI, 0.62–1.55); and where one SNP was homozygous for the mutant alleles (with the other SNP being wildtype) the ORs were 0.86 (95% CI, 0.47–1.58) and 1.19 (95% CI, 0.63–2.25) for *MTHFR* 677C>T and *MTHFR* 1298A>C, respectively. There were no cases identified where both SNPs were homozygous for the mutant allele or both SNPs heterozygous on the same chromosome.

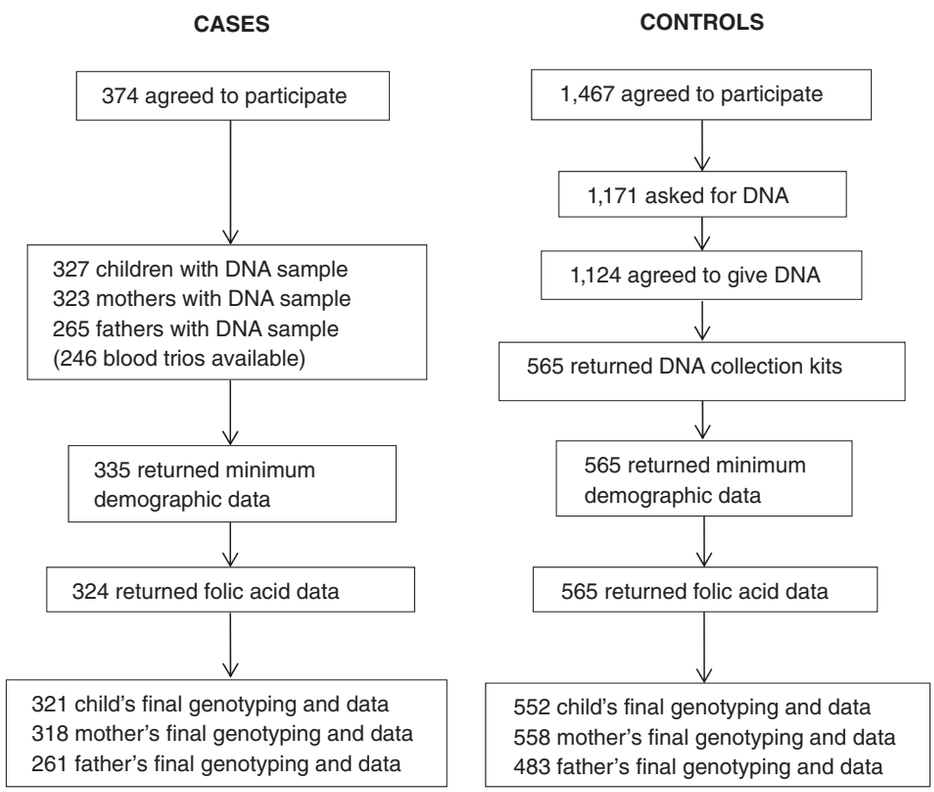

Figure 1.
Flowchart of data collection for folate pathway analysis in the Australian Study of the Causes of Childhood Brain Tumors (Aus-CBT).

Table 1. Demographics of Aus-CBT participants with genotyping and folate data

Variable	Case, *n* (%)	Control, *n* (%)
N child's genotype and covariates available	321	552
Child's age		
0–1	35 (10.9)	62 (11.2)
2–4	90 (28.0)	158 (28.6)
5–9	97 (30.2)	179 (32.4)
10–15	99 (30.8)	153 (27.7)
Child's sex		
Female	132 (41.1)	259 (46.9)
Male	189 (58.9)	293 (53.1)
State of residence		
NSW/ACT	111 (34.6)	167 (30.3)
Victoria/Tasmania	93 (29.0)	156 (28.3)
SA/NT	18 (5.6)	39 (7.1)
WA	42 (13.1)	66 (12.0)
Queensland	57 (17.8)	124 (22.5)
Child's year of birth		
1990–1998	85 (26.5)	132 (23.9)
1998–2003	136 (42.4)	262 (47.5)
2004–2010	100 (31.2)	158 (28.6)
Mother's age at child's birth		
<25	48 (15.0)	35 (6.3)
25–34	200 (62.3)	341 (61.8)
35+	73 (22.7)	176 (31.9)
Maternal education		
<Full high school	78 (24.3)	91 (16.5)
Full high school/trade qualification	110 (34.3)	190 (34.4)
University/college	133 (41.4)	271 (49.1)
Ethnic group[a]		
European	202 (62.9)	429 (77.7)
At least 50% European	73 (22.7)	85 (15.4)
At least 50% non-European	14 (4.4)	17 (3.1)
Indeterminate	32 (10.0)	21 (3.8)
	Mean (SD)	**Mean (SD)**
Child's age	6.8 (4.4)	6.5 (4.4)
Mother's age	30.3 (5.2)	32.0 (4.8)
Father's age	33.3 (5.7)	34.3 (5.5)

Abbreviations: ACT, Australian Capital Territory; NSW, New South Wales; NT, Northern Territory; SA, South Australia; WA, Western Australia.

[a]European = at least 3 European grandparents; 50% European = 2 European grandparents; at least 50% non-European = 2 non-European grandparents and ethnicity of other 2 grandparents unknown; Indeterminate = no 2 grandparents with the same ethnicity (i.e., European or non-European) and 2+ grandparents of unknown ethnicity.

Only low-grade gliomas and embryonal tumors, the two largest CBT subtypes, were analyzed separately due to limited patient numbers for other subtypes. The results for child genotype were generally similar for these two subtypes, with only small differences detected (e.g., for *MTFHR* 677C>T and *MTRR* 66A>G; Supplementary Table S6). Maternal and paternal genotypes also showed little difference between tumor subtypes (Supplementary Tables S7 and S8, respectively).

Among children included in this analysis, the OR for maternal use of folic acid use in the month before pregnancy was 0.63 (95% CI, 0.46–0.87; not shown in tables). Overall, there was minimal evidence that this association varied by genotype, with all but one interaction *P* value being >0.05. Effect estimates tended to lack precision due to small numbers in some cells. There was, however, some evidence of stronger inverse associations when the child or especially the mother carried one or more mutant alleles at *MTRR* 66A>G (Table 3). These associations were not modified by maternal age (*P* value of folic acid by *MTRR* genotype interaction term was 0.06 for mothers both above and below median age of 31, and the folic acid by maternal age interaction term *P* value was 0.93). When folic acid use was stratified by paternal *MTRR* genotype, the pattern of results was similar with ORs for AA, AG, and GG being 1.01 (95% CI, 0.44–2.30), 0.73 (95% CI, 0.44–1.21), and 0.51 (95% CI, 0.25–1.04), respectively (data not tabulated).

Child genotype was also stratified by child sex, results are shown in Supplementary Table S9. There was some statistical evidence of sex differences for CBS 2199 T>C and *MTR* A>G. In addition, the negative association for *MTRR* 66GG seemed weaker in male children, although the suggestive interaction with folic acid use noted for this locus was stronger in males ($P_{\text{interaction}} = 0.06$) compared with females ($P_{\text{interaction}} = 0.48$; results not tabulated).

Discussion

Overall, we observed only a little evidence that folate pathway genotypes are associated with risk of CBT. There was some evidence of a reduced risk among children who had, or whose father had, the *MTRR* 66AG or GG genotype. In addition, the case–parent trio analysis suggested an increased risk associated with the child's carriage of a mutant allele at *CBS* 2199T>C.

Most of the SNPs investigated did not appear to modify the previously described protective association between maternal folic acid use before pregnancy and risk of CBT (1). We observed weak evidence that this association was stronger if the child, mother or father carried one or more mutant alleles at *MTRR* 66A>G; this possible interaction appeared to be stronger in boys.

Folic acid supplementation use is advocated for the prevention of neural tube defects in the months just before pregnancy, such that the mother will have sufficient blood folate levels during conception and the crucial developmental period for neural tube closure in the first few weeks of pregnancy. Even if supplement use is discontinued postconception, red cell folate levels can remain elevated for over 3 months (15), so folate pathway genotypes of the fetus (as well as the mother) could potentially interact with prepregnancy maternal folic acid supplementation. In addition, the genotype of the father may have subtle effects on sperm DNA quality (16) that are insufficient to cause infertility or obvious genetic abnormality, but may increase the likelihood of malignancy. Whether DNA damage in the sperm leads to disease outcome may depend in part on the health and DNA repair capacity of the oocyte (17, 18). Oocyte quality can be influenced by folate availability (19), and so maternal folate levels may determine whether residual DNA damage remains in the fertilized egg.

The two previous studies investigating folate pathway polymorphisms and CBT examined only the child's genotype (6, 7). The Thai study investigated *MTHFR* 677C>T and 1298A>C, *MTR* 2756A>G, thymidylate synthase (*TS*) 28 bp tandem repeat and reduced folate carrier (*RFC*) 80GA. Similar to our findings, these investigators reported no significant association overall between the child's *MTHFR* 677C>T, *MTHFR* 1298A>C or *MTR* 2756A>G genotype and risk of CBT (6). This study reported an increased risk of embryonal tumors associated with the *MTHFR* 1298CC genotype (OR 3.9; 95% CI, 1.3–11.4); the corresponding OR in our study was 1.66 (95% CI, 0.66–4.14), based on 7 cases and 42 controls. Salnikova and colleagues reported a small reduction in CBT risk associated with the *MTHFR* 677CT/TT genotype (the only folate pathway SNP investigated) among boys (OR 0.68; 95% CI, 0.46–1.00) but not girls (OR 1.04; 95% CI, 0.66–1.65; ref. 7). In keeping with our overall results, these investigators also

Table 2. Folate pathway polymorphisms in the child and parents and the risk of CBT

		Child case–control (n = 321/552)[a]		Case–parent trios (n = 246)[a]	Mother case–control (n = 318/558)[a]		Father case–control (n = 261/483)[a]	
Polymorphism	**Genotype**	***n* cases/controls**	**OR[b] (95% CI)**	**OR (95% CI)**	***n* cases/controls**	**OR[b] (95% CI)**	***n* cases/controls**	**OR[b] (95% CI)**
MTHFR 677C>T	CC	143/228	1.0 (Reference)	1.00 (Reference)	129/245	1.0 (Reference)	105/206	1.0 (Referent)
	CT	144/253	0.95 (0.70–1.29)	0.87 (0.62–1.22)	151/251	1.20 (0.88–1.63)	123/209	1.22 (0.87–1.72)
	TT	32/64	0.83 (0.51–1.36)	0.64 (0.36–1.12)	34/51	1.40 (0.84–2.32)	31/60	1.10 (0.65–1.85)
	CT/TT	176/317	0.93 (0.69–1.24)	0.84 (0.60–1.18)	185/302	1.23 (0.92–1.65)	154/269	1.19 (0.86, 1.65)
MTHFR 1298A>C	AA	159/273	1.0 (Reference)	1.00 (Reference)	166/251	1.0 (Reference)	117/243	1.0 (Referent)
	AC	129/227	0.95 (0.70–1.28)	1.17 (0.83–1.65)	126/249	0.72 (0.53–0.98)	122/180	1.30 (0.93–1.82)
	CC	32/42	1.24 (0.73–2.09)	0.93 (0.51–1.71)	25/49	0.81 (0.47–1.40)	20/51	0.81 (0.45, 1.46)
	AC/CC	161/269	0.99 (0.74–1.32)	1.15 (0.82–1.62)	151/298	0.74 (0.55–0.98)	142/231	1.19 (0.86, 1.64)
MTRR 66A>G	AA	80/102	1.0 (Reference)	1.00 (Reference)	66/118	1.0 (Reference)	67/76	1.0 (Referent)
	AG	148/264	0.77 (0.53–1.11)	0.96 (0.66–1.40)	159/245	1.25 (0.85–1.83)	124/242	0.60 (0.40, 0.91)
	GG	90/175	0.71 (0.48–1.07)	0.63 (0.37–1.06)	88/180	0.94 (0.62–1.42)	66/149	0.54 (0.34, 0.87)
	AG/GG	238/439	0.75 (0.53–1.06)	0.91 (0.63–1.33)	247/425	1.12 (0.78–1.61)	190/391	0.58 (0.39, 0.86)
CBS 2199T>C	TT	88/171	1.0 (Reference)	1.00 (Reference)	98/170	1.0 (Reference)	80/130	1.0 (Referent)
	TC	150/246	1.16 (0.83–1.64)	1.42 (0.95–2.14)	143/267	0.91 (0.65–1.27)	131/245	0.81 (0.56, 1.17)
	CC	80/123	1.30 (0.87–1.93)	2.01 (1.20–3.38)	72/107	1.12 (0.75, 1.68)	47/93	0.82 (0.52, 1.32)
	TC/CC	230/369	1.21 (0.88–1.66)	1.49 (1.00–2.23)	215/374	0.97 (0.71–1.33)	178/338	0.81 (0.57, 1.15)
MTR 2756A>G	AA	214/356	1.0 (Reference)	1.00 (Reference)	214/340	1.0 (Reference)	167/311	1.0 (Referent)
	AG	96/165	0.93 (0.68–1.27)	0.90 (0.63–1.30)	91/180	0.80 (0.58–1.09)	76/146	0.90 (0.63–1.29)
	GG	9/26	0.58 (0.26–1.30)	0.84 (0.33–2.14)	10/28	0.55 (0.25–1.18)	16/16	2.04 (0.96–4.30)
	AG/GG	105/191	0.88 (0.65–1.19)	0.90 (0.63–1.30)	101/208	0.76 (0.56–1.03)	92/162	1.01 (0.72, 1.41)
MTR 5049C>A	CC	141/209	1.0 (Reference)	1.00 (Reference)	120/217	1.0 (Reference)	105/158	1.0 (Referent)
	CA	129/260	0.75 (0.54–1.02)	0.82 (0.57–1.18)	150/259	1.03 (0.76–1.42)	117/237	0.77 (0.55–1.10)
	AA	49/76	1.16 (0.75–1.79)	0.81 (0.48–1.37)	45/71	1.34 (0.85–2.10)	38/75	0.76 (0.47, 1.24)
	CA/AA	178/336	0.83 (0.62–1.12)	0.82 (0.57–1.17)	195/330	1.09 (0.81–1.47)	155/312	0.77 (0.55, 1.07)

[a]Numbers are maximum available with genotyping data—sample fails or deletions due to Mendelian inconsistency may result in lower total for individual polymorphisms.

[b]ORs adjusted for child's sex, age, State of residence, year of birth, ethnic group, maternal (paternal) age, and maternal education.

found no overall association between *MTHFR* 677C>T and glial or embryonal tumors (using a dominant model). We are unable to compare our findings for *MTRR* 66A>G or *CBS* 2199T>C with either previous study as they did not investigate these SNPs.

Dietary folate is important not only in the synthesis of DNA but also in the maintenance of DNA methylation levels within cells and it has long been known that methylation of DNA is a key regulator of gene transcription. Aberrations in DNA methylation leading to either hypermethylation or hypomethylation have been strongly linked to the development of human cancer (20). MTRR is a key enzyme in the homocysteine/methionine metabolic pathway and plays a major role in the production of S-adenosyl methionine, which is the sole methyl donor in cells. Importantly, partial loss of enzyme function via a hypomorphic *MTRR* mutation in a murine model has been shown to lead to developmental defects and congenital malformations, highlighting the importance of this enzyme for normal embryonic development (21). The *MTRR* 66GG variant has been shown to produce up to 4-fold lower enzyme activity by comparison with the wild-type allele (22), suggesting a functional role of this polymorphism in affecting the methionine metabolic pathway as well as a mechanistic link with aberrant methylation levels in cells. In addition, *MTRR* 66G has also been linked to a number of cognitive disorders in children (23). In the current study, the reduced OR for the GG genotype was observed across family members and subgroup analyses, and, taken together, the data suggest the *MTRR* 66GG genotype has a protective effect against CBT particularly in situations involving maternal prepregnancy folic acid supplementation. Interestingly, similar protective associations were seen between this genotype and risk of childhood ALL (3, 24–27). This suggests that a mechanism involving this SNP may be common to CBT and some other childhood malignancies; such potential pathways warrant investigation.

The Aus-CBT study has certain strengths. Cases were ascertained from participating oncology centers where virtually all children with CBT in Australia are treated. Structured questionnaires were used to assess maternal folic acid use in specific periods relating to pregnancy, and genotyping performance and concordance was good. We collected DNA from children and both parents, and were able to investigate possible effects of parental genotype and to conduct case–parent trio analyses. We adjusted for all variables found to be confounders of the association between folic acid use and CBT risk in our previous work. Although alcohol is known to interfere with folate metabolism (28, 29), it was not found to be a confounder in our previous analyses (1) and so was not included in the current analyses.

It is also important to note certain limitations of this study. We have previously shown that the control families in our study had higher socioeconomic status than the general population (30). Therefore, although we adjusted for factors that may be related to selection, such as parental education and ethnicity, there may be residual confounding by these factors. In addition, we had a poor return rate (~47%) of DNA samples from controls in our study. However, genotype and related interaction results are unlikely to be affected by selection bias or confounding. We investigated six folate SNPs in three individuals per family, examined interactions of genotypes with maternal use of folic acid, and investigated associations by disease subtype; hence, a large number of ORs were estimated, so some of the observed associations may be due to chance. Also, because of the relatively small numbers (especially when stratifying by sex, folic acid use, or tumor subtype), effect estimates lacked precision; these factors should be considered when interpreting our findings, which should be considered as a point of comparison to future studies.

It is another possible concern that folic acid fortification of wheat flour for bread making became mandatory in Australia in

Table 3. ORs for maternal folic acid supplementation pre-pregnancy and risk of CBT by folate pathway genotypes of child and mother

	Child genotype				Mother genotype			
	N cases/controls				N cases/controls			
Polymorphism	No folic acid[c]	Folic acid	OR[a] (95% CI)	$P_{interaction}$[b]	No folic acid[c]	Folic acid	OR[a] (95% CI)	$P_{interaction}$[b]
MTHFR 677C>T								
CC	100/116	43/112	0.47 (0.29–0.78)	0.33	91/135	38/110	0.60 (0.37–0.98)	0.14
CT	100/156	44/97	0.87 (0.54–1.41)		108/141	43/110	0.52 (0.31–0.85)	
TT	25/44	7/20	0.33 (0.09–1.21)		22/38	12/13	1.32 (0.33–5.34)	
CT/TT	125/200	51/117	0.82 (0.54–1.27)	0.16	130/179	55/123	0.65 (0.42–1.00)	0.51
MTHFR 1298A>C								
AA	113/160	46/113	0.68 (0.43–1.07)	0.98	116/147	50/104	0.68 (0.43–1.07)	0.79
AC	93/113	36/94	0.60 (0.36–1.01)		93/142	33/107	0.46 (0.27–0.78)	
CC	20/21	12/21	0.53 (0.15–1.93)		15/25	10/24	0.78 (0.23–2.67)	
AC/CC	113/154	48/115	0.59 (0.37–0.95)	0.97	108/167	43/131	0.53 (0.33–0.84)	0.61
MTRR 66A>G								
AA	52/66	28/36	0.89 (0.44–1.79)	0.18	42/72	24/46	1.00 (0.47–2.11)	0.02
AG	109/153	39/111	0.54 (0.33–0.88)		109/144	50/101	0.80 (0.50–1.28)	
GG	64/96	26/79	0.50 (0.27–0.94)		70/95	18/85	0.26 (0.14–0.51)	
AG/GG	173/249	65/190	0.57 (0.39–0.83)	0.07	179/239	68/186	0.54 (0.38–0.78)	0.10
CBS 2199T>C								
TT	60/93	28/78	0.64 (0.35–1.18)	0.97	68/89	30/81	0.56 (0.31–1.00)	0.31
TC	106/146	44/100	0.62 (0.38–1.01)		103/152	40/115	0.58 (0.36–0.93)	
CC	58/74	22/49	0.59 (0.28–1.23)		49/69	23/38	1.03 (0.49–2.17)	
TC/CC	164/220	66/149	0.65 (0.44–0.96)	0.95	152/221	63/153	0.68 (0.46–1.00)	0.68
MTR 2756A>G								
AA	151/200	63/156	0.62 (0.42–0.92)	0.94	149/185	65/155	0.53 (0.36–0.80)	0.74
AG	69/103	27/62	0.72 (0.39–1.34)		67/109	24/71	0.64 (0.35–1.19)	
GG	6/13	3/13	– (–)		7/18	3/10	– (–)	
AG/GG	75/116	30/75	0.71 (0.40–1.26)	0.77	74/127	27/81	0.71 (0.40–1.28)	0.46
MTR 5049C>A								
CC	103/121	38/88	0.53 (0.32–0.89)	0.60	85/121	35/96	0.57 (0.34–0.97)	0.98
CA	89/154	40/106	0.77 (0.47–1.27)		107/151	43/108	0.65 (0.40–1.05)	
AA	33/39	16/37	0.56 (0.23–1.39)		30/40	15/31	0.59 (0.23–1.51)	
CA/AA	122/193	56/143	0.72 (0.47–1.10)	0.40	137/191	58/139	0.65 (0.43–0.98)	0.90

[a]ORs adjusted for child's sex, age, State of residence, year of birth, ethnic group, maternal age, and maternal education.
[b]*P* value of polymorphism by folic acid interaction term in a model where main effects accounted for.
[c]"No folate" prepregnancy is the reference for all ORs.

September 2009, relevant legislation having been passed in 2007. While it is plausible that fortification of bread with folic acid would influence any effect of use of folic acid supplements on risk of CBT, no cases or controls in our study were conceived, and only two were born, after September 2009. No official data on incremental introduction of fortification between July 2007 and September 2009 are available; however, only 13 cases and 22 controls in our study were conceived after July 2007.

In conclusion, this study revealed only a little evidence that folate pathway genotypes are associated with risk of CBT. The *MTRR* 66GG genotype in child, mother, or father, particularly when accompanied by maternal folic acid supplementation pre-pregnancy, appeared to be associated with a reduced risk of CBT, however, this result needs to be replicated in larger studies or pooled CBT datasets.

Disclosure of Potential Conflicts of Interest

N.G. Gottardo reports receiving a commercial research grant from Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R.J. Scott, J. Attia, C. Bower, N.H. de Klerk, F.M. van Bockxmeer, L.J. Ashton, B.K. Armstrong, E. Milne
Development of methodology: R.J. Scott, C. Bower, N.H. de Klerk, M.D. Norris, M. Haber, L.J. Ashton, B.K. Armstrong, E. Milne
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.J. Scott, F.M. van Bockxmeer, N.G. Gottardo, E. Milne
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.R. Greenop, C. Bower, N.H. de Klerk, S.E. Jamieson, B.K. Armstrong, E. Milne
Writing, review, and/or revision of the manuscript: K.R. Greenop, R.J. Scott, J. Attia, C. Bower, N.H. de Klerk, M.D. Norris, M. Haber, S.E. Jamieson, F.M. van Bockxmeer, N.G. Gottardo, L.J. Ashton, B.K. Armstrong, E. Milne
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.R. Greenop, E. Milne
Study supervision: R.J. Scott, J. Attia, C. Bower, E. Milne

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Kathryn R. Greenop, Rodney J. Scott, John Attia, et al.

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