

Folate Pathway Gene Polymorphisms, Maternal Folic Acid Use, and Risk of Childhood Acute Lymphoblastic Leukemia

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Abstract

Background: Several studies suggest that maternal folic acid supplementation before or during pregnancy protects against childhood acute lymphoblastic leukemia (ALL). We investigated associations between ALL risk and folate pathway gene polymorphisms, and their modification by maternal folic acid supplements, in a population-based case-control study (2003–2007).

Methods: All Australian pediatric oncology centers provided cases; controls were recruited by national random digit dialing. Data from 392 cases and 535 controls were included. Seven folate pathway gene polymorphisms (*MTHFR* 677C>T, *MTHFR* 1298A>C, *MTRR* 66A>G, *MTR* 2756 A>G, *MTR* 5049 C>A, *CBS* 844 Ins68, and *CBS* 2199 T>C) were genotyped in children and their parents. Information on prepregnancy maternal folic acid supplement use was collected. ORs were estimated with unconditional logistic regression adjusted for frequency-matched vari-

ables and potential confounders. Case-parent trios were also analyzed.

Results: There was some evidence of a reduced risk of ALL among children who had, or whose father had, the *MTRR* 66GG genotype: ORs 0.60 [95% confidence interval (CI) 0.39–0.91] and 0.64 (95% CI, 0.40–1.03), respectively. The ORs for paternal *MTHFR* 677CT and TT genotypes were 1.41 (95% CI, 1.02–1.93) and 1.81 (95% CI, 1.06–3.07). ORs varied little by maternal folic acid supplementation.

Conclusions: Some folate pathway gene polymorphisms in the child or a parent may influence ALL risk. While biologically plausible, underlying mechanisms for these associations need further elucidation.

Impact: Folate pathway polymorphisms may be related to risk of childhood ALL, but larger studies are needed for conclusive results. *Cancer Epidemiol Biomarkers Prev*; 24(1); 48–56. ©2014 AACR.

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Introduction

Acute lymphoblastic leukemia (ALL), the most common childhood malignancy, has a significant genetic basis for susceptibility (1–4). As well as contributing to the risk of childhood ALL, inherited genetic variation appears to predispose to specific ALL subtypes and response to chemotherapeutic intervention (2). As most cases of childhood ALL are thought to be initiated *in utero* (5), etiologic research has focused on dissecting the complex interaction between genetic susceptibility and fetal environmental exposures. Following the publication of findings from an Australian study suggesting that maternal folic acid supplementation during pregnancy protects against childhood common ALL (6), there has been growing interest in the possible role of folic acid and folate pathway genes. If folate is associated with risk of ALL, then it is biologically plausible that polymorphisms in these genes would also be associated with its risk.

Polymorphisms of the methylene tetrahydrofolate reductase (*MTHFR*) gene at 677C>T or 1298A>C are known to reduce enzyme activity, which is essential for the bioavailability and metabolism of folate (7, 8). Since 2001, more than 20 studies have reported on the association between the child's *MTHFR* genotype and risk of childhood ALL, and seven meta-analyses have been published (9–15). The results of these studies vary considerably,

but the most recent studies report either no association with either polymorphism (13) or a protective effect of *MTHFR* 677C>T alone (14).

Other folate gene polymorphisms that have been studied in relation to childhood ALL are 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*; refs. 16–23), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*; refs. 17, 22, 24–28), methylenetetrahydrofolate dehydrogenase (*MTHFD1*; refs. 22, 25, 27, 29), thymidylate synthetase (*TS/TYMS*; refs. 17, 20, 22, 25, 29–32), and reduced folate carrier (*RFC1*; refs. 26, 27, 30, 32). A recent meta-analysis including *MTRR* and *MTR* polymorphisms indicated a possible protective effect of *MTRR* 66GG genotype (13).

The inconsistency in results of individual studies is attributed, at least partly, to limited sample sizes and ethnicity-related differences in allele frequencies in the populations. The impact of folate gene polymorphisms may also vary depending on folate status (33, 34). A French Canadian study found some combinations of *MTHFR* 677C>T and 1298A>C polymorphisms protected against childhood ALL only among children born before folic acid supplementation recommendations commenced (1998; ref. 35). Lupo and colleagues (21) also reported a similar difference before or after U.S. folate fortification in 1996 for maternal *MTR* genotype on ALL risk. Thus, differing folate status in populations may explain some of the discrepant results; it has been recommended that studies of folate polymorphisms and ALL risk should take account of folic acid intake (9, 14, 36).

Two studies have examined variation in folate pathway polymorphisms by maternal folic acid use. A Californian study reported interactions between maternal folic acid use and filial genotype at specific loci on cystathionine-beta-synthase (*CBS*), *MTRR*, and *TYMS* genes (25), while a French study found no evidence of such interactions with polymorphisms in the *MTHFR* or *MTRR* genes (24).

While most previously published studies of folate pathway gene polymorphisms have reported results for the child's genotype only, it is possible that the parents' genotype may also be important in determining fetal folate status. For example, several studies suggest that the risk of folate-related cardiac birth defects is affected by maternal genotype in folate pathway genes (37–40).

In the Australian Study of the Causes of ALL (Aus-ALL), which was specifically powered to investigate the association between maternal folic acid supplementation and risk of ALL, we did not replicate the previous strong association between folic acid use during pregnancy and ALL risk (6). However, weak associations were seen with use pre-pregnancy; ORs were 0.84 [95% confidence interval (CI), 0.55–1.28] for 300.1 to 450 µg per day, and 0.77 (95% CI, 0.52–1.14) for >450 µg per day (41).

Our primary aim was to investigate associations between filial and parental folate pathway gene polymorphisms and risk of ALL, including by ALL subtype where possible. Our second aim was to investigate whether maternal use of folic acid pre-pregnancy modified any observed associations of folate pathway polymorphisms with ALL.

Materials and Methods

Aus-ALL was a national, population-based, multicenter case-control study conducted in Australia between 2003 and 2007. The study design and data collection methods have been described elsewhere (41, 42). Briefly, incident ALL cases were identified

through all 10 Australian pediatric oncology centers, where the large majority of cases are treated. Inclusion criteria were: initial remission achieved, biological mother available and able to complete questionnaires in English, and Australian residence. Controls were prospectively recruited in the same time period by national random digit dialing (RDD), and frequency matched to cases by age (within 1 year), sex, and state of residence. For the genetic component of Aus-ALL, controls were frequency matched to cases on age, sex, and state of residence in a ratio of approximately 1.5:1. The study was approved by the Human Research Ethics Committees at all participating hospitals; parents of participating children provided written informed consent.

Parents were mailed questionnaires about key environmental and demographic variables. Mothers completed a questionnaire detailing folic acid supplement use with dose in the 3 months before and during pregnancy. Blood samples for DNA analysis were collected from case children and their parents at the treating hospital, while buccal cell samples were collected from control children and their parents at home using FTA cards (43). Case children also provided a buccal sample using this method. Punches taken from the cards were prepared for PCR as previously described (43), and samples were then subjected to whole genome amplification using the GenomiPhi V2 DNA Amplification Kit (GE Healthcare) according to the manufacturer's instructions.

Case and control children and their parents were genotyped for seven polymorphisms in folate pathway genes: *MTHFR* 677C>T (rs1801133), *MTHFR* 1298A>C (rs1801131), *MTRR* 66A>G (rs1801394), *CBS* 844 Ins68, *CBS* 2199T>C (rs706208), *MTR* 2756A>G (rs1805087), *MTR* 5049C>A (rs2853523).

The *MTHFR* 677C>T and *MTHFR* 1298A>C polymorphisms were analyzed as previously described (44). For *MTRR* 66A>G, *CBS* 2199T>C and *CBS* 844 Ins68 polymorphisms, genotyping was performed on an ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems). The *MTR* 2756A>G and *MTR* 5049C>A polymorphisms were analyzed using a modification of the method of Morrison and colleagues (45).

For quality assurance purposes, 10% of samples were selected at random for repeat analysis within laboratories, and this was performed blinded in each laboratory. Between-laboratory concordance was also assessed at *MTHFR* 677C>T. Concordance between case children's genotypes derived using blood and WGA'd buccal samples was also examined.

Statistical analysis

Mendelian inconsistencies were identified in family data using the PedCheck software (46); 119 results from 28 case families and 76 control families were excluded. Hardy-Weinberg Equilibrium (HWE) analysis was performed in STATA v10 using unaffected, unrelated individuals. No polymorphisms were found to significantly deviate from HWE (data not shown).

The results of the quality assurance analyses were excellent with genotyping call rate 99%, within-laboratory concordance 99.7%, and between-laboratory concordance 98%. However, quality assurance analysis of genotypes derived from WGA'd buccal DNA samples showed discrepancies of 2.3% to 7.4% with those derived from lymphocyte DNA. Therefore, we applied a statistical adjustment method (using discordance estimates empirically derived from quality-control data in cases) in all our analyses. This method and the rationale for it have been described in detail elsewhere (47). Briefly, this method applies misclassification

probabilities to measured data, and then combines the results using 50 multiple imputations to minimize bias in the ORs.

We had previously seen weak evidence of an inverse association with ALL risk when mothers took folic acid ≥ 300 $\mu\text{g}/\text{day}$ in the 3 months pre-pregnancy (41). We therefore categorized folic acid use for analysis as ≥ 300 $\mu\text{g}/\text{day}$ versus none pre-pregnancy. As red cell folate levels are maintained for 3 to 4 months (48), this dose was also relevant to early pregnancy (90% of women taking folic acid pre-pregnancy also reported use in the first trimester). ORs and 95% CIs for main effects of each polymorphism were estimated using unconditional logistic regression analysis in R version 2.14.2 (2012, The R Foundation for Statistical Computing; www.r-project.org/). All case-control analyses were adjusted for the frequency matching factors—age, sex and state of residence. To ensure models were comparable, and because folic acid use may be confounded by some variables, all variables meeting the empirical criteria for confounding (independently associated with both the outcome and folic acid supplementation in the controls) were included in genotype models. They were birth order, ethnicity, maternal age, and parental education. Additional analyses were conducted for particular immunophenotypes and cytological subtypes of ALL (each of these models included all controls). To see whether there were different associations of genotypes with ALL in mothers who did and did not take folic acid, the main effects analyses of genotypes were repeated separately for these two groups.

Statistical interactions were assessed on the basis of the effect size and significance of the interaction term in the model (Wald test), and the resulting change in main effect estimates due to the presence of the interaction term. The interaction *P* values from these models are shown in the text, where appropriate, and results from corresponding stratified analyses are shown in the tables.

Case-parent trio analyses were undertaken using conditional logistic regression in StataIC version 11 (StataCorp) and genotype-based ORs were estimated. This method uses the genotypes of the parents to create pseudosiblings of case children using all possible genotypes the offspring could have had as controls that are matched on all environmental variables (i.e., no confounding variables need to be included as pseudosiblings have the same parents as cases; ref. 49). Data were prepared using "pseudoc" and "gtab" commands developed by David Clayton (50).

The results for all analyses were similar when children with birth defects (22 cases and 17 controls) were excluded, so these children were included in the final models.

Results

We were notified of 568 incident cases of ALL; 49 were ineligible to participate (three with no biological mother available 30 had mothers with insufficient English, 12 were nonresident, and four did not reach remission). Of the 519 eligible cases, parents of 416 (80.2%) consented to participate in the study, and genotyping results were obtained from 392 case children, 391 case mothers, and 344 case fathers. Child-parent genotyping trios were available for 276 case families.

Of the 2,947 eligible control families identified through RDD, 2,071 (70.3%) agreed to take part in the main study (41). In accordance with requirements for age and sex frequency matching, 1,067 control families were asked to provide DNA for the genetic component of the study; of these, 1,027 (96%) agreed. In all, genotyping results were available from 535 (50%) control

Table 1. Demographic characteristics of cases and controls in the Australian Study of causes of acute lymphoblastic leukemia in children, 2003–2007

N with child genotype	Cases	Controls
Variable	392	535
	n (%)	n (%)
Sex		
Male	176 (44.9)	252 (47.1)
Female	216 (55.1)	283 (52.9)
Age group		
0–1.9	35 (8.9)	43 (8.0)
2–4.9	178 (45.4)	239 (44.7)
5–9.9	111 (28.3)	182 (34.0)
10–14.9	68 (17.3)	71 (13.3)
State of residence		
NSW/ACT	123 (31.4)	164 (30.7)
Victoria/Tasmania	117 (29.8)	156 (29.2)
SA/NT	43 (11.0)	48 (9.0)
WA	40 (10.2)	61 (11.4)
Queensland	69 (17.6)	106 (19.8)
Birth order		
1	186 (47.4)	220 (41.1)
2	120 (30.6)	181 (33.8)
3+	86 (21.9)	134 (25.0)
Child's birth year		
1988–1993	54 (13.8)	62 (11.6)
1994–1999	127 (32.4)	207 (38.7)
2000–2006	211 (53.8)	266 (49.7)
Higher level of education of either parent		
Nontertiary	223 (56.9)	253 (47.3)
Tertiary	169 (43.1)	282 (52.7)
Ethnicity ^a		
European	282 (71.9)	427 (79.8)
At least 50% European	79 (20.2)	79 (14.8)
At least 50% non-European and not known if 50% European	14 (3.6)	11 (2.1)
Indeterminate	17 (4.3)	18 (3.4)
Household income		
<\$20,000	28 (7.2)	21 (3.9)
\$20,001–40,000	67 (17.2)	73 (13.9)
\$40,001–70,000	115 (29.6)	185 (34.6)
\$70,001–100,000	93 (23.9)	137 (25.7)
>\$100,000 pa	86 (22.1)	117 (22.9)
Mother's age at child's birth		
<25 years	59 (15.1)	44 (8.2)
25–34 years	266 (67.9)	360 (67.3)
35+ years	67 (17.1)	131 (24.5)
Child had a birth defect		
Yes	22 (5.7)	17 (3.2)
Folic acid ^b		
None	261 (67.4)	326 (61.6)
>0–300 μg	44 (11.4)	50 (9.5)
300.1–450 μg	37 (9.6)	61 (11.5)
450+ μg	45 (11.6)	92 (17.4)

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

^aEuropean = 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.

^bMaternal folic acid supplementation in the 3 months prior to the pregnancy.

children, 501 (47%) control mothers, and 447 (42%) control fathers. Supplementary Figure S1 shows the flow chart for DNA and data collection.

The distributions of demographic variables among cases and controls included in the analysis were generally similar, with some exceptions (Table 1). Case children were more likely to be first

born than controls and to have a birth defect. Control parents were more likely than case parents to be tertiary educated, have a higher income and to have been 35 years of age or older when the index child was born.

Overall, few associations were seen between folate pathway polymorphisms and risk of ALL. There was some evidence of reduced risk associated with the *MTRR* 66GG genotype in the child and the father, but not the mother (Table 2). ORs for *MTHFR* 677CT and TT genotypes were elevated for paternal genotype only. The case–parent trio analyses were mostly consistent with those of the case–control analysis of the child's genotype with two exceptions: *MTHFR* 1298CC and *MTR* 2756 GG (Table 2). The results of the case–control analysis were similar when restricted to the 276 children belonging to case–parent trios (data not shown).

Associations with the child's genotype were also examined for the main immunophenotypes and cytogenetic subtypes (Table 3). The inverse association with the *MTRR* 66GG genotype was observed for most ALL subtypes, whereas the ORs for *CBS* 844 Ins68 were elevated among cases with hyperdiploidy or Trisomy 21, and reduced among cases with *ETV6*-*Runx-1* t(12, 21). *MTHFR* 677TT appeared to be positively associated with risk of T-cell ALL. The results for maternal and paternal genotype across subtypes seemed largely consistent with the overall findings (Supplementary Tables S1 and S2, respectively).

Results of gene polymorphisms stratified by folic acid supplementation are shown in Table 4 (for *MTHFR* and *MTRR*) and Supplementary Table S3 (for *MTR* and *CBS*). Evidence for gene–folic acid interactions was weak with all but one interaction *P* value being >0.05. ORs for *MTRR* 66GG genotype in the child were reduced irrespective of maternal folic acid use (Table 3; interaction *P* value = 0.35), whereas the OR for maternal *MTRR* 66GG genotype appeared reduced only among mothers who took ≥ 300 μ g folic acid/day; however, the interaction *P* value was 0.17. There was some evidence that filial or maternal carriage of at least one T allele at *MTHFR* 677C>T was associated with an increased risk of ALL if the mother took ≥ 300 μ g folic acid/day, but not otherwise (interaction *P* values 0.04 for the child's genotype and 0.09 for maternal genotype). Child's age, sex, and birth order were similar between those children who provided DNA and those who did not. However, in those who had provided DNA, mothers were older (92% vs. 82% were 25+), and parental education and income levels were higher (53% vs. 37% had tertiary education; 18% vs. 35% had annual income <\$40,000), and more mothers used folic acid prepregnancy (36% vs. 23%; all *P* < 0.001). The above figures were similar where the control father had provided DNA. The median folic acid dose for the analyzed controls was 450 μ g/day (interquartile range, 200) which was the same as the entire group previously reported (41).

Discussion

Our study of folate pathway gene polymorphisms and their association with ALL risk was the first to report results for polymorphisms in the child, mother, and father; this is important to have a comprehensive view of the potential role of genetic susceptibility in disease risk, and its source(s). Both case–control and case–parent trio analyses of associations with the child's genotype were undertaken, yielding largely consistent results. We also investigated possible interactions between child and mater-

nal genotype and maternal use of folic acid in the preconception period.

Overall, we found little evidence of associations between folate pathway genotypes and risk of childhood ALL, or that maternal folic acid use in the period leading up to the pregnancy modified associations between genotype and ALL risk. However, there was some evidence of a reduced risk among children who had, or whose father had, the *MTRR* 66AG or GG genotype. In addition, paternal *MTHFR* 677TT genotype appeared to be associated with an increased risk, while there was also weak evidence of possible interactions between maternal prepregnancy use of folic acid and *MTHFR* 677CT/TT genotypes in the child and/or mother. Finally, the results of our subtype analyses suggested that some folate genotypes in the child may be associated with risk of specific disease subtypes.

Consistent with our findings for the child's *MTRR* 66A>G genotype, four of seven previous studies reported at least some evidence of a protective effect of the mutant allele (24, 26–28). In addition, a meta-analysis of three studies assessing this polymorphism (26–28) reported summary ORs for AG and GG of 0.76 (95% CI, 0.60–0.96) and 0.67 (95% CI, 0.52–0.88), respectively (13). For the *MTHFR* genotypes, however, a number of previous studies suggested that 677C>T may be protective, but that 1298A>C was not associated with risk (14, 15). We did not find evidence of a reduced risk with either polymorphism overall, similar to two recent studies (22, 24).

Seven out of eight previous studies investigating the child's *MTR* 2756A>G genotype and risk of childhood ALL reported null or inconclusive findings (16, 18, 20, 25–28), similar to our study. Only the UK Childhood Cancer Study reported elevated ORs: 1.24 (95% CI, 1.00–1.53) and 1.88 (95% CI, 1.16–3.07) for the AG and GG genotypes, respectively (19). Vijayakrishnan and Houlston (13) reported a null result in their meta-analysis of four studies (18, 26–28). The weight of the evidence would therefore suggest no association of the *MTR* 2756A>G genotype and risk of childhood ALL. No previous studies of childhood ALL and *MTR* 5049C>A, *CBS* 844 ins68, or *CBS* 2199T>C genotypes were found.

Three previous studies have investigated interactions between selected folate pathway polymorphisms and maternal folic acid supplementation. In our case-only analysis of 83 ALL cases from Western Australia (diagnosed between 1984 and 1992), we found no evidence of interactions with the child's *MTHFR* 677C>T or 1298A>C genotype (51). Metayer and colleagues (25) recently reported evidence of interactions between maternal folic acid use and specific loci on the *CBS*, *MTRR*, and *TYMS* genes; however, these loci were not investigated in our study. A recent French study (24) reported no interactions, although results were not presented in the article.

Only three previous studies have investigated maternal folate pathway genotype in relation to ALL risk; two found that the child's genotype was more closely associated with risk than the mother's (19, 35), while Lupu and colleagues found more associations with the mother's than the child's genotype (17). In our study, the findings for the child's genotype were generally consistent with those for maternal genotype. We were unable to identify any previous studies of paternal genotype with which to compare our findings.

Two previous studies have reported results for the child's folate pathway genotype by immunophenotype. The UKCCS reported null associations with *MTHFR* 677C>T and 1298A>C for both B- and T-cell ALL (19), while Tong and colleagues reported evidence

Table 2. Main effects of child's, mother's, and father's folate pathway genotypes on risk of childhood ALL

N cases/controls Polymorphism	Child genotype			Mother genotype			Father genotype		
	Case-control analysis			Case-parent trios			Case-control analysis		
	N cases/controls ^a	Genotype	OR ^b (95% CI)	N cases/controls ^a	OR (95% CI)	N cases/controls ^a	OR ^b (95% CI)	N cases/controls ^a	OR ^c (95% CI)
MTHFR 677 C>T (rs180133)	166/244	CC	1.00 (reference)	176/212	1.00 (reference)	131/204	1.00 (reference)	170/188	1.41 (1.02-1.93)
	181/221	CT	1.22 (0.92-1.63)	110 (0.80-1.51)	0.90 (0.67-1.20)	170/188	0.90 (0.67-1.20)	170/188	1.41 (1.02-1.93)
	43/60	TT	1.23 (0.77-1.97)	0.92 (0.54-1.56)	115 (0.71-1.86)	41/43	1.15 (0.71-1.86)	41/43	1.81 (1.06-3.07)
	224/281	CT/TT	1.23 (0.94-1.62)	1.08 (0.79-1.48)	0.94 (0.71-1.24)	213/280	0.94 (0.71-1.24)	213/280	1.48 (1.09-2.00)
	195/264	AA	1.00 (reference)	1.00 (reference)	1.00 (reference)	159/213	1.00 (reference)	159/213	1.00 (reference)
MTHFR 1298 A>C (rs180131)	169/192	AC	1.04 (0.78-1.38)	0.77 (0.57-1.05)	178/203	0.98 (0.73-1.31)	156/164	1.09 (0.80-1.49)	
	27/52	CC	0.98 (0.54-1.79)	0.49 (0.28-0.87)	35/47	1.38 (0.79-2.42)	25/44	1.06 (0.56-2.01)	
	196/244	AC/CC	1.02 (0.78-1.35)	0.74 (0.55-1.01)	213/250	1.03 (0.77-1.37)	181/208	1.08 (0.80-1.47)	
	88/101	AA	1.00 (reference)	1.00 (reference)	81/97	1.00 (reference)	82/75	1.00 (reference)	
MTR 66A>G (rs1801394)	208/233	AG	0.84 (0.58-1.22)	0.95 (0.66-1.38)	195/229	0.82 (0.56-1.21)	160/204	0.62 (0.41-0.93)	
	88/174	GG	0.60 (0.39-0.91)	0.71 (0.44-1.16)	105/143	0.94 (0.61-1.44)	88/129	0.64 (0.40-1.03)	
	296/407	AG/GG	0.75 (0.53-1.06)	0.92 (0.63-1.32)	300/372	0.87 (0.60-1.24)	248/333	0.62 (0.42-0.93)	
	251/337	AA	1.00 (reference)	1.00 (reference)	253/302	1.00 (reference)	219/274	1.00 (reference)	
	130/158	AG	1.00 (0.75-1.34)	1.12 (0.79-1.58)	118/142	0.86 (0.64-1.17)	106/119	0.93 (0.67-1.29)	
MTR 2756 A>G (rs1805087)	10/19	GG	1.70 (0.52-5.58)	0.47 (0.19-1.19)	19/29	1.82 (0.73-4.56)	12/19	1.93 (0.58-6.35)	
	140/177	AG/GG	1.03 (0.78-1.37)	1.07 (0.76-1.52)	137/171	0.92 (0.69-1.23)	118/138	0.98 (0.71-1.34)	
	155/199	CC	1.00 (reference)	1.00 (reference)	161/181	1.00 (reference)	131/160	1.00 (reference)	
	181/229	CA	0.98 (0.72-1.32)	1.12 (0.80-1.57)	172/212	0.92 (0.68-1.25)	159/182	1.06 (0.76-1.49)	
MTR 5049 C>A (rs285523)	55/83	AA	1.04 (0.67-1.61)	1.27 (0.76-2.13)	56/71	1.09 (0.69-1.70)	49/64	1.14 (0.70-1.86)	
	236/312	CA/AA	1.01 (0.76-1.33)	1.13 (0.82-1.58)	228/283	0.96 (0.72-1.28)	208/246	1.08 (0.79-1.49)	
	118/147	TT	1.00 (reference)	1.00 (reference)	110/143	1.00 (reference)	110/123	1.00 (reference)	
	179/253	TC	0.82 (0.59-1.13)	0.92 (0.65-1.29)	188/238	0.95 (0.68-1.32)	166/200	0.89 (0.62-1.27)	
CBS 2199 T>C (rs706208)	88/105	CC	1.23 (0.82-1.84)	1.34 (0.84-2.15)	85/85	1.47 (0.96-2.26)	53/81	0.81 (0.50-1.31)	
	267/358	TC/CC	0.94 (0.69-1.28)	0.96 (0.69-1.35)	273/323	1.08 (0.79-1.47)	219/281	0.88 (0.63-1.24)	
	450/329	WTWT	1.00 (reference)	1.00 (reference)	336/409	1.00 (reference)	288/360	1.00 (reference)	
CBS 844 Ins 68 ^d	55/69	WTIns/InsIns	0.79 (0.53-1.17)	1.03 (0.62-1.72)	49/71	0.67 (0.44-1.01)	48/69	0.67 (0.44-1.01)	

^aNumbers are from raw data before correction imputation method applied, individual polymorphism Ns may vary from total due to typing fails.
^bORs adjusted for matching variables (child's age, sex, and state of residence), mother's age group, parental education, birth order, and ethnicity.
^cORs adjusted for matching variables (child's age, sex, and state of residence), father's age group, parental education, birth order, and ethnicity.
^dN for homozygous mutants insufficient to produce separate OR.

Table 3. Child's folate pathway genotypes and risk of ALL by immunophenotype and cytogenetic subtype

Polymorphism	Genotype	PreB cell (n = 346)	T cell (n = 37)	ETV6-Runx-1 [t(12, 21); (n = 61)]	Hyperdiploidy (n = 115)	Trisomy or higher 21 (n = 67)
		OR ^a (95% CI)	OR ^a (95% CI)	OR ^a (95% CI)	OR ^a (95% CI)	OR ^a (95% CI)
MTHFR 677 C>T	CC	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
	CT	1.21 (0.90–1.63)	1.39 (0.63–3.06)	0.96 (0.53–1.72)	1.48 (0.95–2.30)	2.19 (1.22–3.93)
	TT	1.11 (0.68–1.83)	2.57 (0.92–7.19)	0.42 (0.12–1.52)	0.86 (0.37–1.98)	0.73 (0.20–2.65)
	CT/TT	1.19 (0.90–1.58)	1.63 (0.78–3.39)	0.85 (0.48–1.50)	1.37 (0.89–2.11)	1.95 (1.10–3.47)
MTHFR 1298 A>C	AA	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
	AC	1.00 (0.74–1.35)	1.58 (0.74–3.36)	1.41 (0.78–2.56)	0.81 (0.52–1.28)	1.05 (0.58–1.87)
	CC	0.87 (0.46–1.63)	1.20 (0.29–4.91)	1.00 (0.29–3.39)	1.18 (0.55–2.56)	1.27 (0.47–3.45)
	AC/CC	0.98 (0.74–1.31)	1.51 (0.73–3.12)	1.35 (0.76–2.40)	0.86 (0.56–1.32)	1.07 (0.62–1.85)
MTRR 66A>G	AA	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
	AG	0.83 (0.57–1.21)	0.87 (0.33–2.30)	1.25 (0.57–2.74)	0.77 (0.45–1.34)	0.84 (0.41–1.73)
	GG	0.58 (0.37–0.89)	0.99 (0.33–2.95)	0.61 (0.24–1.55)	0.46 (0.24–0.90)	0.63 (0.27–1.44)
	AG/GG	0.73 (0.51–1.05)	0.88 (0.35–2.26)	0.99 (0.46–2.11)	0.65 (0.38–1.10)	0.79 (0.40–1.58)
MTR 2756 A>G ^b	AA	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
	AG/GG	1.08 (0.80–1.44)	0.63 (0.28–1.39)	1.20 (0.66–2.17)	0.95 (0.61–1.49)	1.25 (0.71–2.19)
MTR 5049 C>A	CC	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
	CA	0.99 (0.72–1.35)	1.21 (0.57–2.62)	1.24 (0.68–2.27)	1.04 (0.65–1.67)	1.09 (0.59–1.99)
	AA	1.05 (0.67–1.65)	0.71 (0.19–2.66)	0.51 (0.16–1.61)	1.34 (0.70–2.55)	1.25 (0.53–2.94)
	CA/AA	1.01 (0.75–1.35)	1.12 (0.54–2.35)	1.07 (0.60–1.92)	1.11 (0.71–1.73)	1.14 (0.64–2.03)
CBS 2199 T>C	TT	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
	TC	0.80 (0.57–1.12)	1.23 (0.50–3.03)	0.93 (0.47–1.84)	0.81 (0.50–1.32)	0.58 (0.32–1.05)
	CC	1.25 (0.82–1.89)	1.37 (0.46–4.10)	1.07 (0.45–2.54)	0.92 (0.50–1.72)	0.32 (0.12–0.85)
	TC/CC	0.92 (0.67–1.26)	1.30 (0.55–3.07)	0.98 (0.52–1.87)	0.87 (0.55–1.37)	0.53 (0.30–0.92)
CBS 844 Ins 68 ^b	WTWT	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
	WTIns/InsIns	0.80 (0.53–1.19)	0.99 (0.37–2.67)	0.26 (0.08–0.89)	1.63 (0.97–2.73)	1.61 (0.83–3.13)

^aAll ORs adjusted for matching variables (child's age, sex, state of residence), maternal age, child ethnicity, birth order, parental education, and corrected for genotype method discordance.

^bN for homozygous mutants insufficient to produce separate OR.

of a protective association between *MTHFR* 677TT genotype and both ALL subtypes (52). We found no evidence of associations between either SNP and B-cell ALL, and only weak evidence that the *MTHFR* 677TT genotype was associated with an elevated risk of T-cell ALL. The UKCCS reported ORs of 1.82 (95% CI, 1.09–3.03) and 1.63 (95% CI, 0.55–4.88) for B- and T-cell ALL (respectively) associated with *MTR* 2756GG genotype (19), while we found no association with either type of ALL. The UK study also found that *MTHFR* 677TT was associated with an increased risk, and the 1298CC genotype a decreased risk, of the ETV-Runx-1 cytogenetic subtype (19), while we observed no evidence of either association with this ALL subtype.

As previously suggested, the inconsistency among published results for folate pathway polymorphisms is probably due to the small sample sizes in most studies and the fact that both genotype frequencies and folate status vary among study populations.

A biologic role for polymorphisms in folate metabolizing genes in the etiology of ALL is certainly plausible, despite some of the inconsistent findings reported in this area. The folate pathway is critical to cancer development, being involved in DNA synthesis, repair, and methylation (53), and low folate status and/or aberrant folate metabolism are known to potentiate disease development (54). Polymorphisms in folate pathway genes cause altered enzyme kinetics and thereby alter folate availability; for example, polymorphisms in genes coding for folate metabolic enzymes have been shown to affect the synthesis of S-Adenosyl methionine and result in hyper- and hypomethylation states of DNA (55). Thus, folate pathway gene polymorphisms may contribute to disease susceptibility through epigenetic regulation of gene expression involving DNA hypomethylation (56).

Our finding of a potential increased risk of ALL associated with paternal *MTHFR* 677TT genotype is consistent with what is known about the impact of this polymorphism on *MTHFR* activity. Thus, relative to individuals with the wild-type *MTHFR* 677CC genotype, those with the TT genotype have lower levels of genomic DNA methylation (57). In mice, sperm hypomethylation has been shown to increase disease susceptibility in the offspring (58), and there is growing evidence from human studies that paternal folate status can affect metabolic gene expression in the offspring through epigenetic mechanisms involving chromatin packaging in sperm (59, 60). Such mechanisms may explain our findings for paternal *MTHFR* 677C>T, but it is not clear why similar overall associations were not seen with the child's or mother's genotype. We did observe elevated ORs for ALL with the *MTHFR* 677C>T genotype in the child and mother when the mother took ≥ 300 μ g folic acid in the prepregnancy period. As folic acid supplementation would at least partly counter the tendency to hypomethylation associated with carriage of the mutant allele at *MTHFR* 677C>T, and therefore mitigate the potentially increased risk of ALL, the possible mechanisms underlying these findings are not clear and require further investigation.

The *MTRR* gene is involved in the folate metabolic cycle, and reactivates oxidized cobalamin–MTR complex (which itself catalyzes the remethylation of homocysteine to methionine) by reductive methylation (54). It is not clear why the *MTRR* 66GG genotype should have a protective association with ALL; however, alterations in the activity of *MTRR* are likely to have a profound effect on epigenetic programming which may explain a protective effect due to a reduction in the ability to provide methyl groups required for hypermethylation (61).

Table 4. ORs for folate pathway genotypes stratified by maternal use of folic acid (≥ 300 μg) in the 3 months before pregnancy

N cases/controls	Polymorphism	Folic acid	Genotype	Child (354/495 ^a)		Mother (353/461 ^a)				
				n case/control ^a	OR ^b (95% CI)	n case/control ^a	OR ^b (95% CI)			
MTHFR 677 C>T		None	CC	117/144	1.0 (reference)	127/129	1.0 (reference)			
			CT	115/134	1.03 (0.72-1.50)	104/137	0.74 (0.51-1.08)			
			TT	27/41	0.94 (0.52-1.71)	28/33	1.02 (0.55-1.90)			
			CT/TT	142/175	1.03 (0.72-1.45)	132/170	0.80 (0.56-1.13)			
		≥ 300 μg	CC	32/80	1.0 (reference)	32/63	1.0 (reference)			
			CT	50/67	1.92 (1.07-3.43)	48/75	1.26 (0.69-2.29)			
			TT	11/19	1.63 (0.65-4.11)	12/15	2.01 (0.77-5.24)			
			CT/TT	61/86	1.85 (1.06-3.23)	60/90	1.36 (0.77-2.41)			
			MTHFR 1298A>C		None	AA	130/164	1.0 (reference)	119/139	1.0 (reference)
						AC	111/116	1.03 (0.71-1.49)	114/131	0.86 (0.59-1.26)
CC	19/36	0.90 (0.43-1.89)				27/26	1.69 (0.78-3.68)			
AC/CC	130/152	1.01 (0.71-1.43)				141/157	0.94 (0.65-1.36)			
≥ 300 μg	AA	47/83			1.0 (reference)	42/69	1.0 (reference)			
	AC	43/61			1.09 (0.62-1.90)	44/58	1.10 (0.60-2.00)			
	CC	3/13			0.64 (0.14-2.98)	6/17	0.89 (0.27-2.95)			
	AC/CC	46/74			1.05 (0.61-1.82)	50/75	1.08 (0.61-1.91)			
	MTRR 66A>G				None	AA	57/63	1.0 (reference)	56/61	1.0 (reference)
						AG	135/146	0.89 (0.55-1.45)	126/148	0.73 (0.45-1.19)
GG			63/104	0.74 (0.43-1.27)		71/81	1.05 (0.60-1.82)			
AG/GG			198/250	0.82 (0.51-1.31)		197/229	0.84 (0.53-1.33)			
≥ 300 μg			AA	24/32	1.0 (reference)	22/29	1.0 (reference)			
			AG	51/69	0.65 (0.32-1.34)	50/65	0.74 (0.36-1.54)			
			GG	17/60	0.34 (0.15-0.79)	19/51	0.47 (0.20-1.10)			
			AG/GG	68/129	0.53 (0.27-1.06)	69/116	0.65 (0.33-1.30)			

^aAll numbers are before genotype correction imputation (cases/controls where mother took >0 and <300 μg folic acid are excluded, Ns for individual polymorphisms may vary due to sample fails).

^bORs adjusted for matching variables, mother's age group, parental education, birth order, and ethnicity, and each main effect corrected for genotype error.

Aus-ALL has both strengths and limitations. Cases were ascertained from participating oncology centers where virtually all children with ALL in Australia are treated, and 75% of eligible cases participated in Aus-ALL. Only four cases did not reach remission and were therefore ineligible for inclusion. We have previously shown that the control families in our study had higher socioeconomic status than the general population, and that those who returned the questionnaires and provided DNA samples were higher still (42). In addition, DNA was provided from only 51% of families from whom it was requested, and these families tended to be of higher SES, have older mothers, and be more likely to use folic acid. Therefore, although we adjusted for factors that may be related to selection and/or to completion of questionnaires and provision of DNA, such as parental education and ethnicity, there may be residual confounding by these factors. While these differences should not influence the main analysis of folate pathway genes, or the gene by folic acid use interactions, it may increase the frequency of folic acid users in the control group. Because of the dependence on self-reported data, there is likely to have been misclassification of folic acid use. Misclassification, however, is likely to have been nondifferential because we used standardized questionnaires and little is known in the community about a possible relationship between folic acid and risk of ALL.

Some of the observed associations may be due to chance. We investigated seven folate gene polymorphisms in three individuals, examined interactions of genotypes with maternal use of folic acid and did subgroup analyses by ALL immunophenotype and cytogenetic subtypes; hence, a large number of ORs were estimated. Buccal samples were used for controls, and statistical correction of known blood-buccal discordance was required for these genotypes, which adds to the potential imprecision of results. Estimates also lacked precision because of the relatively

small numbers in the subgroup analyses; these factors should be considered when interpreting our findings. Some associations, however, were consistently observed across family members and subgroup analyses, and are concordant with most previous studies; for example, those with *MTRR* 66A>G.

In conclusion, our findings suggest that some folate pathway polymorphisms in the child and parents are related to risk of childhood ALL, and that some of these associations vary by maternal folate status. The apparent lack of an overall protective effect of the *MTHFR* 667C>T polymorphism against ALL in our study, in contrast with most previous studies, may reflect adequacy of dietary folate and supplemental folic acid intake in Australia (62, 63). In light of growing evidence of a paternal contribution to disease susceptibility in the offspring, further studies of paternal genotype are needed. Pooling data from similar studies internationally would substantially strengthen our ability to investigate folate gene-environment interactions in the etiology of childhood ALL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Consortium Statement

The Aus-ALL consortium conducted the study and the Telethon Kids Institute, University of Western Australia, was the coordinating centre. Bruce Armstrong (Sydney School of Public Health), Elizabeth Milne (Telethon Kids Institute), Frank van Bockxmeer (Royal Perth Hospital), Michelle Haber (Children's Cancer Institute Australia), Rodney Scott (University of Newcastle), John Attia (University of Newcastle), Murray Norris (Children's Cancer Institute Australia), Carol Bower (Telethon Kids Institute), Nicholas de Klerk (Telethon Kids Institute), Lin Fritschi (WA Institute for Medical Research), Ursula Kees (Telethon Kids Institute), Margaret Miller (Edith Cowan University), and Judith

Thompson (WA Cancer Registry) were the research investigators and Helen Bailey (Telethon Kids Institute) was the project coordinator. The clinical Investigators were: Frank Alvaro (John Hunter Hospital, Newcastle); Catherine Cole (Princess Margaret Hospital for Children, Perth); Luciano Dalla Pozza (Children's Hospital at Westmead, Sydney); John Daubenton (Royal Hobart Hospital, Hobart); Peter Downie (Monash Medical Centre, Melbourne); Liane Lockwood (Royal Children's Hospital, Brisbane); Maria Kirby (Women's and Children's Hospital, Adelaide); Glenn Marshall (Sydney Children's Hospital, Sydney); Elizabeth Smibert (Royal Children's Hospital, Melbourne); and Ram Suppiah (previously Mater Children's Hospital, Brisbane).

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BLOOD CANCER DISCOVERY

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