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The Folate Pathway and Nonsyndromic Cleft Lip and Palate

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Abstract

Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a common birth malformation caused by genetic, environmental and gene-environment interactions. Periconceptional supplementation with folic acid, a key component in DNA synthesis and cell division, has reduced the birth prevalence of neural tube defects (NTDs) and may similarly reduce the birth prevalence of other complex birth defects including NSCLP. Past studies investigating the role of two common methylenetetrahydrofolate reductase (MTHFR) SNP polymorphisms, C677T (rs1801133) and A1298C (rs1801131), in NSCLP have produced conflicting results. Most studies of folate pathway genes have been limited in scope, as few genes/SNPs have been interrogated. In this study, we asked whether variations in a more comprehensive group of folate pathway genes were associated with NSCLP and, if so, were there detectable interactions between these genes and environmental exposures. In addition, we evaluated the data for a sex effect. Fourteen folate metabolism related genes were interrogated using eighty-nine SNPs in multiplex and simplex non-Hispanic White (NHW) (317) and Hispanic (128) NSCLP families. Evidence for a risk association between NSCLP and SNPs in nitrous oxide 3 (NOS3) and thymidylate synthetase (TYMS) was detected in the NHW group, whereas associations with methionine synthase (MTR), betaine-homocysteine methyltransferase (BHMT2), MTHFS and SLC19A1 were detected in the Hispanic group. Evidence for over-transmission of haplotypes and gene interactions in the methionine arm was detected. These results suggest that perturbations of the genes in the folate pathway may contribute to NSCLP. There was evidence for an interaction between several SNPs and maternal smoking, and for one SNP with sex of the offspring. These results provide support for other studies that suggest that high maternal homocysteine levels may contribute to NSCLP and should be further investigated.

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Keywords

Nonsyndromic cleft lip and palate; NSCLP; folate metabolism; association; genetics; homocysteine; methionine

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCLP) is a common isolated congenital birth defect affecting 4000 newborns annually, ranking it the fourth most common birth anomaly in the United States (Hashmi et al., 2005; Wyszynski, 2002). Epidemiological and family studies suggest that NSCLP is a complex disorder caused by both genetic and environmental factors (Christensen, 1999; Schutte and Murray, 1999). Identification of genes contributing to this disorder has been challenging, but steady progress has uncovered a number of susceptibility genes, including IRF6, TGF α , TGF β 3, MSX1 and CRISPLD2 (Chiquet et al., 2007a; Field et al., 2004; Jia et al., 2009; Marazita et al., 2002; Marazita et al., 2004; Schutte and Murray, 1999). Environmental agents such as alcohol, smoking, pharmaceutical compounds and diet have inconsistently been implicated as etiologic factors (Jia et al., 2009; Jugessur et al., 2003; Krapels et al., 2006; Lie et al., 2008; Shaw et al., 2009; Shi et al., 2008; Wyszynski, 2002).

Folate deficiency has been causally related to a number of birth defects, especially neural tube defects (NTDs). This observation led to the fortification of grain products in 1998, with a concurrent decrease in NTDs (Blom et al., 2006; Botto et al., 2006; Boyles et al., 2006; Canfield et al., 2005). However, the underlying mechanisms by which folic acid prevents NTDs remains to be defined (Blom et al., 2006; Boyles et al., 2006). The neural tube and the craniofacial regions both arise from neural crest cells, leading to the hypothesis that folic acid deficiency may also contribute to nonsyndromic clefting (Badovinac et al., 2007; Chevrier et al., 2007). Population-based studies have not shown an overall decrease in orofacial clefts since dietary folate fortification; in addition, numerous genetic studies have not found an association with the common C677T and A1298T polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene (Blanton et al., 2000; Blanton et al., 2002; Botto et al., 2006; Canfield et al., 2005; Hashmi et al., 2005; Jugessur et al., 2003; Mostowska et al., 2006a; Shaw et al., 2006). Nonetheless, folic acid and vitamin supplementation in high-risk NSCLP pregnancies does show that recurrence in families can be reduced (Badovinac et al., 2007; Tolarova and Harris, 1995; Wilcox et al., 2007). The results of these latter studies suggest a beneficial effect of prenatal folic acid dietary supplementation, particularly with regard to the risk of recurrence of NSCLP.

Folate metabolism is a complex process which depends on a series of enzymatic reactions involving numerous genes and pathways that form derivatives of THF (tetrahydrofolate), which is the active form of folic acid. A variety of intermediates are produced and function in vital physiological processes such as nucleotide synthesis, cell division, and tissue growth (Blom et al., 2006; Boyles et al., 2006; Sharp and Little, 2004). Perturbation of any part of the interacting folate metabolism pathways could result in a frank folate deficiency, with the downstream effect of disrupting important biological processes, such as craniofacial

development (Fig. 1). In contrast, high levels of homocysteine may also affect developmental activities such as neural crest cell motility and migration, which are important in early development (Brauer and Tierney, 2004).

MTHFR is the key enzyme for producing the active form of folate (5-methyl-THF) that is required by cells (Fig. 1). Two polymorphisms, C677T and A1298C, that decrease enzymatic activity are the most commonly studied variants in the folate pathway with respect to NSCLP (Blanton et al., 2000; Blanton et al., 2002; Boyles et al., 2008b; Brandalize et al., 2007; Chevrier et al., 2007; Martinelli et al., 2006; Mostowska et al., 2006a; Pezzetti et al., 2004; Prescott et al., 2002). Previously, we and others found no evidence for an association with either variant in different NSCLP populations (Blanton et al., 2000; Blanton et al., 2002; Boyles et al., 2008b; Brandalize et al., 2007). Other genes in the folate pathway have been interrogated to determine whether variation in these genes was associated with NSCLP. Evidence for an association has been found for SNPs in NOS3, CBS and BHMT (Boyles et al., 2008a; Lammer et al., 2005; Mostowska et al., 2010; Rubini et al., 2005a).

The majority of studies to date have evaluated individual folate pathway genes, specific parts of the pathway or only a few SNPs in the genes of interest. A comprehensive evaluation of the folate pathway examining both individual SNP variation and gene interactions should provide information about whether variation in these genes contributes to NSCLP. Therefore, we evaluated SNPs in 14 genes in or interacting with the folate pathway, in order to identify variations associated with NSCLP risk and assessed them for smoking and gender effects.

METHODS

Study population and sample preparation

The study sample was composed of 120 multiplex NSCLP families (83 non-Hispanic white and 37 Hispanic) and 325 simplex parent-child trios (234 non-Hispanic white and 91 Hispanic) which have been extensively described previously (Chiquet et al., 2007a). Briefly, families were ascertained by a proband with NSCLP from three craniofacial centers: Children's Hospital, Boston, Texas Children's Hospital in Houston, and the University of Texas Craniofacial Clinic at Houston (Hecht et al., 1991a; Hecht et al., 1991b). Cases were examined and all families with syndromic clefting were excluded from analysis. Ethnicity was self-reported and all Hispanic cases were of Mexican ancestry. Blood or saliva samples were collected after obtaining informed consent and DNA was extracted using either the Roche DNA Isolation Kit for Mammalian Blood (Roche, Switzerland), or the Oragene Purifier kit for saliva (DNA Genotek Inc., Ontario, Canada) following the manufacturer's protocol.

Ninety-seven SNPs in 14 folate pathway related genes were selected based on heterozygosity (>0.3), functionality, inter- and intragenic positions, total coverage of the gene, and tagging ability as previously described (Table 1)(Chiquet et al., 2007a). The SNPs were genotyped using the SNplex Genotyping System [Applied Biosystems (ABI), Foster City, CA] and detected with the 3730 DNA Analyzer (ABI). Allele calls were determined

using GeneMapper analysis software (ABI). The data was then imported into Progeny Lab (South Bend, IN, USA) and checked for Mendelian inconsistencies using the PedCheck program (O'Connell and Weeks, 1998).

Statistical Methods

The data was stratified by ethnicity initially and then further stratified by family history. Hardy-Weinberg equilibrium (HWE) and allele frequency differences were evaluated using SAS (v9.1). Pair-wise linkage disequilibrium values (D' and r^2) were calculated using GOLD and parametric and non-parametric linkage analyses were conducted using MERLIN (Abecasis et al., 2002; Abecasis and Cookson, 2000). Parametric linkage parameters have been previously described (Chiquet et al., 2008). Evidence for association was tested using Pedigree Disequilibrium Test (PDT), Geno-PDT and Association in the Presence of Linkage (APL) test (Chung et al., 2006; Martin et al., 2000; Martin et al., 2006). Generalized Estimating Equations (GEE) as implemented in SAS (v8) using PROC GENMOD was used to examine gene-gene interactions of SNPs (Hancock et al., 2007). The minor allele was considered to be the risk allele in all cases. Only two-way interactions were tested with GEE because of sample size. Gene-environment interactions with sex and smoking were evaluated with FBAT-I under an additive model (Hoffmann et al., 2009). One million iterations were used for p-value generation. Evaluations were not conducted in the Hispanic dataset due to sample size.

In silico analyses were performed to identify the potential functionality of associated SNPs located in regulatory regions. Analyses were conducted using three online programs which employ different algorithms: Alibaba2, Patch and Transcription Element Search Software (TESS) (Grabe, 2002; Matys et al., 2006; Schug, 2003). Allele sequences were submitted to the programs and the outputs for all allelic variants were compared.

RESULTS

Ninety-seven SNPs in 14 genes from the folate pathway (Figure 1), MTHFR, MTR, MTRR, MTHFD1, MTHFD2, MTHFS, TYMS, FOLR1, FOLR2, SLC19A1, BHMT, BHMT2, CBS and NOS3, were genotyped in our NHW and Hispanic families. Seven SNPs with call rates of <95% were excluded from the analysis. One SNP was not in HWE and was removed from further analysis. This resulted in a total of eighty-nine SNPs in the final analysis (Table 1). Allele frequencies for thirty of the eighty-nine SNPs differed between the NHW and Hispanic groups (Table 1); therefore the data was stratified by ethnicity. In addition, the data was further stratified by the presence or absence of family history (FH) of NSCLP (Lewis et al., 1987).

There was no evidence for linkage by parametric and nonparametric analyses in either ethnic group (data not shown). Significant intragenic linkage disequilibrium was found in both datasets ($r^2 > 0.95$).

No association between NSCLP and either of the common MTHFR variants (C677T/rs1801133 and A1298C/rs1801131) or any of the other SNPs in MTHFR was found in the NHW group (Supplemental Table 1). Nominal evidence of altered transmission was found

for twenty-one SNPs in eight other folate related genes ($0.006 \leq p < 0.05$) across all data subsets and analyses (Table 2A). All genes except for MTHFS had at least 2 SNPs with altered transmission. In most cases, there was altered transmission in the aggregate families and either the positive family history group or the negative family history group. For example, the strongest association was seen for rs502396/TYMS in the aggregate families and there was altered transmission in the negative family history group but not the positive family history group. In contrast, rs2373929/NOS3 had altered transmission in the aggregate families and a similar association was seen in the positive family history group. One SNP (rs828858/MTHFD2) had altered transmission in only the positive family history group while four SNPs had altered transmission in only the negative family history group.

After Bonferroni correction for the number of unlinked genes (12), none of the altered transmissions were significant ($p < 0.004$). However, considering that there were eighty-nine SNPs interrogated, by chance there should be approximately four with a $p \leq 0.05$. In the overall PDT, there were nine SNPs with $p < 0.05$, more than twice as many as expected by chance ($p = 0.02$).

In the Hispanic dataset, twenty-two SNPs in nine genes had altered transmission (Table 2B). Four of these SNPs were in MTHFR and included one of the common functional variants (A1298C/rs1801131, $p = 0.029$). Six of the genes also had SNPs with altered transmission in the NHW dataset (MTR, BHMT2, BHMT, MTHFS, TYMS, CBS). Unlike the NHW associations, most of the evidence for altered transmission came from the positive family history group; only six of the associated SNPs did not have altered transmission in this subset.

SNPs in two genes (rs1422086/BHMT2; rs2115540/MTHFS) remain significant in the positive family history group after Bonferroni correction (Table 2B). In addition, there are fourteen SNPs in the PDT analysis of the positive family history group with $p < 0.05$, three times as many as expected by chance alone ($p = 0.0001$).

Results of the 2-SNP haplotype analyses are presented by ethnicity in Table 3. For the NHW group, one BHMT2 haplotype, rs476620-rs626105, and one NOS3 haplotype, rs1800779-rs2373929, showed altered transmission ($p = 0.002$ for each) (Table 3). Only the NOS3 SNPs had individually altered transmission (Table 2). Altered transmission of haplotypes in four genes, BHMT, NOS3, MTHFS and TYMS, was observed in the Hispanic group (Table 3). One SNP from each of the haplotypes also had individually altered transmission (Table 2).

Evidence for numerous gene interactions was found in both groups and is summarized in Table 4. The same five gene interactions, albeit with different SNPs, were seen in both populations (BHMT2-CBS, MTHFD1-SLC19A1, MTHFR-SLC19A1, NOS3-CBS, TYMS-CBS). In the NHWs, thirteen genes were involved in thirty-two gene interactions irrespective of individual SNPs; SNPs in seven of these genes had altered transmission individually. CBS (5) and MTHFD1 (5) interacted with the largest number of different genes. In the Hispanic group, thirteen genes were involved in thirty-four gene interactions. SNPs in eight of these genes had altered transmission individually. CBS (6) and SCL19A1 (5) interacted with the largest number of different genes. Again, as with the single SNP

analysis, most interactions do not survive a Bonferroni correction based on the number of genes ($p < 0.0047$); only the FOLR1/MTHFS interaction surpasses this in the NHW group and none in the Hispanic dataset. However, based on the number of gene interactions tested (105), we would expect only one p-value less than 0.01, while there are thirty-two and thirty-four in the NHW and Hispanic groups, respectively ($p < 0.00001$ for each).

A single SNP (rs651646) in FOLR2 showed a significant sex interaction ($p = 0.0006$) in our NHW dataset. While no SNPs in FOLR2 were identified as being associated with NSCLP in the single SNP analyses, FOLR2 SNPs were involved in several gene interactions.

Significant interactions were identified in the NHW dataset between smoking and four SNPs in two different genes, FOLR1 and FOLR2: two are downstream and two are intronic (Table 5). None of these SNPs were associated with NSCLP in our single SNP or haplotype analyses. However, gene interactions were found for FOLR1 and FOLR2 with MTHFR in the Hispanic group and for FOLR1 and CBS, MTHFD1, MTHFS and SLC19A1 and for FOLR2 and CBS, MTHFS and NOS3 in the NHW group.

Three different transcription factor binding site prediction algorithms were used to evaluate the ancestral and alternative sequences for nineteen SNPs in potential promoter or enhancer regions of thirteen genes (Table 6). The SNPs included in this analysis were identified by single, haplotype and gene interaction analyses. Numerous potential DNA binding sites were identified; in the majority, the alternative allele was predicted to change the binding site. Consistency in the prediction was found for five SNPs in four genes, BHMT2, MTHFD1, MTRR and NOS3. Functional testing is needed to determine the role of these SNPs in gene regulation.

DISCUSSION

Folate supplementation in pregnancy has been shown to reduce the recurrence of NSCLP in families and to have a modest reduction in birth prevalence on a population basis (Hashmi et al., 2005; Krapels et al., 2006; Shaw et al., 1995; Wilcox et al., 2007). However, little is known about the protective mechanism of periconceptional folate supplementation. In this study, we interrogated 14 folate pathway genes for association and gene interactions in our well characterized dataset of NHW and Hispanic multiplex and simplex NSCLP families.

Under strict Bonferroni correction for multiple testing, most of our reported associations/interactions would not be considered significant. However, it is unlikely that there are only one or two variations that lead to an increased susceptibility to NSCLP but rather it is a perturbation of multiple genes in one or more pathways that is important. Thus, we would not expect only one or two SNPs with extremely small p-values. What we actually find is many more nominal p-values than expected by chance alone. Moreover, we have obtained very similar results between our two ethnically diverse samples. However, these results need to be validated in a variety of populations.

In the single SNP analysis, evidence for an association was found for SNPs in eight genes in the NHWs and for nine genes in the Hispanic families. Three of these genes, CBS, MTR, and BHMT2, participate directly in the methionine cycle; several others, including

SLC19A1, MTRR and NOS3, influence this cycle. Fourteen of the individually associated SNPs in ten genes have potential promoter/enhancer roles. In addition, haplotype and gene interaction analyses identified SNPs in three other genes, which have potential regulatory roles. Two noncoding TYMS variants, rs502396 and rs1001761, had altered transmission in the NHW NSCLP families. These SNPs are in introns 1 and 2, respectively, and could be potential enhancer elements. The ancestral allele for rs502396 was predicted to be a DNA binding site, while the rs1001761 ancestral allele was not. However, the alternative alleles for each SNP were predicted to create new DNA binding sites that could modulate the expression of this gene. TYMS is a key enzyme in the production of dihydrofolate, an essential precursor molecule to thymine, which in turn is necessary for DNA synthesis (Abbott et al., 1993; Assaraf et al., 2006; Boyles et al., 2008b; Brauer and Tierney, 2004; Kim et al., 2006; Shuey et al., 1994). Studies of folate antagonists in the treatment of malignancies have shown that, by inhibiting TYMS, DNA synthesis is disrupted and cell cycle arrest is induced (Abbott et al., 1993; Assaraf et al., 2006). This finding suggests that perturbation of TYMS function could affect cell and tissue growth during development (Abbott et al., 1993; Shuey et al., 1994).

SNPs in BHMT2, MTR and CBS in the methionine arm of the folate pathway gave evidence of an association in the Hispanic families. One SNP in BHMT2 (rs626105), located in intron 1, had altered transmission in these families. The ancestral allele was not predicted to be a DNA binding site; however, the alternate allele creates a new site. BHMT2 is a member of the methionine cycle that is ubiquitously expressed; BHMT2 mRNA expression in the fetus has only been found in the liver and kidney (Chadwick et al., 2000). However, these results may reflect the instability of the BHMT2 protein, which is rapidly degraded making it difficult to study (Chadwick et al., 2000; Li et al., 2008). Little is known about the function of BHMT2 other than its role in the remethylation of homocysteine (Li et al., 2008). Perturbation of gene expression with the creation of a new DNA binding site could result in an increase in homocysteine levels leading to hyperhomocysteinemia.

Hyperhomocysteinemia has been implicated as a risk factor for other congenital anomalies such as NTDs, heart defects, and neurocristopathies (Brauer and Tierney, 2004; Rosenquist et al., 1996; Selhub, 2008; Zetterberg, 2004). Hyperhomocysteinemia could either directly or indirectly disrupt a multitude of cellular processes important to lip and palate development including cellular proliferation, apoptosis, migration and DNA synthesis (Brauer and Tierney, 2004; Knott et al., 2003; Zetterberg, 2004). Indeed, in a handful of studies, high plasma homocysteine levels have been observed in mothers after the birth of an infant with a clefting abnormality (Knott et al., 2003; Rubini et al., 2005b; Verkleij-Hagoort et al., 2007; Wong et al., 1999). These latter studies must be interpreted with caution, as they included only a small number of case mothers and were retrospective (Knott et al., 2003; Rubini et al., 2005b; Verkleij-Hagoort et al., 2007; Wong et al., 1999).

Two MTR SNPs, rs4077829 and rs6668344, in introns 9 and 14, respectively, had altered transmission in the negative family history Hispanic group ($p=0.003$ and $p=0.006$ respectively.). It is unknown how these SNPs would affect gene function. Nevertheless, it is interesting that MTR functions in a parallel pathway to BHMT2, and links the folate and methionine cycles, where it re-methylates homocysteine to methionine. Therefore, deficiencies in MTR may affect homocysteine levels similarly to BHMT2, but may also

affect the formation of the recycled one-carbon intermediate THF from 5m-THF. Thus, perturbation of the MTR function(s) could dramatically affect different pathways. Our results, while implicating this gene, require further validation. A specific mechanism for the effect of high levels of homocysteine being restricted to an isolated birth defect is unknown. However, one possibility is the reduction of available cysteine, which is required by CRISPLD2, a gene we have identified as playing a role in palatal formation (Chiquet et al., 2007b).

Sex was the first obvious risk factor documented for NSCLP, with nearly twice as many males affected as females. However, the mechanism for this differential risk remains unknown. We identified only one SNP in FOLR2 which interacts with sex to impact risk. It is unclear why variation in this gene would be sex-influenced. Importantly, this is the first report to include sex as a covariate in examining genes involved in folate metabolism.

In addition, we found evidence for interactions with smoking and SNPs in two genes (FOLR1 and FOLR2). Smoking has been implicated as a risk factor and been shown to lower the levels of folic acid and increase homocysteine (Jauniaux et al., 2007; Ozerol et al., 2004). Elevated levels of homocysteine and its conversion to homocysteine-thiolactone have been suspected of contributing to the post-translational modification of the folate receptors (Jakubowski et al., 2009). Such modification makes the folate receptor a neo-antigen and illicit a maternal autoantibody response, which may further limit folate uptake and make the embryo more susceptible to complex birth defects (Bille et al., 2010; Cabrera et al., 2008). Neither of these genes was identified in the single gene association analyses. Additional studies are needed to determine whether smoking or tobacco metabolites affect expression of these genes.

The three previous studies that had the most comprehensive evaluation of folate related genes generally examined only a few intragenic SNPs per gene and did not include upstream and downstream variants. This is important because upstream variation has been associated with NSCLP (Rahimov et al., 2008). In general, few associations were identified in any of the studies. A SNP in CBS was reported to reduce the risk of NSCLP in the initial Norwegian study; this was not replicated in their subsequent study (Boyles et al., 2008a; Boyles et al., 2009). In addition to the single SNP associations, SNPs in CBS were found to interact with SNPs in a number of genes (BHMT2, FOLR1, FOLR2, MTHFD1, MTHFR, MTRR, NOS3, SLC19A1 and TYMS). Importantly, the interactions with BHMT2 and NOS3 were present in both the NHW and Hispanic groups. Mostowska and colleagues found that a SNP in BHMT (rs3733890) significantly lowered the risk of NSCLP (Mostowska et al., 2006b; Mostowska et al., 2010). This same SNP was examined in the current study and modest evidence for association was found in the Hispanic aggregate families and the positive family history group. In addition, we did identify significant gene-gene interactions involving other SNPs in BHMT (Table 4).

Interpretation of results is especially challenging in complex disorders. Strict Bonferroni correction would exclude many of our findings. Using less conservative approaches, genes in the folate pathway are implicated. A recent GWAS of NSCLP in trios of Asian and European ancestry identified associations with SNPs near MAFB (20q12) and ABCA4

(1p22.1) (Beaty et al., 2010). The authors did not report any suggestive results for SNPs in or near folate pathway genes. This is not surprising, as GWAS are generally underpowered for identifying associations to low penetrance alleles (Badner, 2010). Methods, as performed here, that take a “pathway” approach to analysis may be more successful in identifying the lower risk genes (Linghu et al., 2009).

Altogether, the results of this study suggest that the perturbation of the methionine pathway is associated with an increased risk for NSCLP. Moreover, it provides support for studies suggesting that hyperhomocysteinemia may play an etiologic role (Knott et al., 2003; Ozerol et al., 2004; Rubini et al., 2005b; Verkleij-Hagoort et al., 2007; Wong et al., 1999). Notably, we interrogated only a subset of the genes in the folate and related pathways and there are other genes in this complex set of pathways that were not addressed in this study. These genes and validation of our findings should be the focus of future NSCLP studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Folic acid gene and methionine gene pathways

Genes interrogated in this study are shaded. Substrates abbreviations are the following: MTHFD1/2, methylenetetrahydrofolate dehydrogenase 1 and 2; MTHFS, methenyltetrahydrofolate synthetase; TYMS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase; FOLR1/2, folate receptor 1 and 2; SLC19A1, solute carrier family 19 member 1, also known as reduced folate carrier protein 1; MTR, methionine synthase; MTRR, methionine synthase reductase; BHMT/2, betaine-homocysteine methyltransferase; CBS, cystathionine-beta-synthase; NOS3, nitric oxide synthase; THF, tetrahydrofolate; DHF, dihydrofolate; SHMT, serine hydroxymethyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

Table 1

Folate Pathway Genes: SNP Locations, Alleles, and Ethnic Frequencies

Gene ^a	SNP	Chromosomal location (bp) ^b	Alleles ^c	Location ^b	Amino acid change	NHW MAP ^d	HCE ^e	p value
<i>BHMT</i> 5q13.1-q15 20.4kb	rs645112	78438303	A/C	Upstream 5.1kb		0.422	0.403	0.535
	rs567754	78452172	C/T	Intron 4		0.325	0.399	0.013
	rs3733890	78457715	G/A	Exon 6	R239Q	0.319	0.389	0.018
	rs585800	78462964	A/T	3' UTR		0.294	0.152	<0.0001
	rs617219	78465350	A/C	Downstream 1.5kb		0.336	0.394	0.055
<i>BHMT</i> 2 5q13 19.7kb	rs1915706	78471967	C/T	Downstream 8.1kb		0.354	0.486	0.00001
	rs2253262	78387392	C/A	Upstream 13.9kb		0.372	0.353	0.543
	rs476620	78390202	A/G	Upstream 11.1kb		0.394	0.422	0.355
	rs626105	78405657	G/A	Intron 1		0.221	0.233	0.654
	rs682985	78409187	T/C	Exon 2	Synonymous	0.401	0.437	0.241
<i>CBS</i> 21q22 3 23.1kb	rs1422086	78410621	A/C	Intron 2		0.407	0.449	0.175
	rs234785	43377074	C/G	Upstream 7.6kb		0.330	0.280	0.088
	rs234784	43376503	C/T	Upstream 7.0kb		0.373	0.391	0.572
	rs234783	43376312	C/T	Upstream 6.8kb		0.476	0.651	<0.0001
	rs234714	43361102	C/T	Intron 4		0.237	0.528	<0.0001
<i>FOLR1</i> 11q13.3-q14.1 6.74kb	rs234713	43360960	A/G	Intron 4		0.315	0.170	<0.0001
	rs2851391	43360473	T/C	Intron 4		0.543	0.371	<0.0001
	rs2298759	43359173	A/G	Intron 5		0.440	0.609	<0.0001
	rs2298758	43358596	G/A	Exon 7	Synonymous	0.002	0.011	0.050
	rs11700812	43353659	C/T	Exon 12	R369H	0.016	0.007	0.290
<i>FOLR2</i> 11q13.3-q13.5 5.15kb	rs10512319	43346936	C/G	3' UTR		0.142	0.141	0.958
	rs3016432	71574903	C/T	Upstream 3.3kb		0.467	0.332	<0.0001
	rs2071010	71578612	G/A	Intron 1		0.055	0.071	0.274
	rs1540087	71579139	C/T	Intron 1		0.020	0.022	0.87
	rs11235462	71586273	T/A	Downstream 1.3kb		0.120	0.159	0.063
<i>FOLR2</i> 11q13.3-q13.5 5.15kb	rs651646	71607174	T/A	Intron 1		0.452	0.347	0.001
	rs514933	71607855	A/G	Intron 2		0.369	0.293	0.024
	rs2298444	71610062	A/G	Intron 4		0.166	0.196	0.197

Gene ^a	SNP	Chromosomal location (bp) ^b	Alleles ^c	Location ^b	Amino acid change	NHW MAP ^d	HCF [#]	p value
<i>MTHFD1</i> 14q24 71.6kb	rs2276048	71618860	A/G	Downstream 8.2kb		0.174	0.235	0.001
	rs1076991	63924794	T/C	Upstream 0.052kb		0.450	0.490	0.160
	rs4902283	63954365	C/T	Exon 7	P162L	0.007	0.004	0.530
	rs1885031	63957808	A/G	Intron 8		0.106	0.205	<0.0001
	rs11551058	63963854	G/T	Exon 12	G392W	0.001	0.000	0.570
	rs2236225	63978598	C/T	Exon 20	R653Q	0.467	0.528	0.047
	rs2236224	63978904	C/T	Intron 20		0.404	0.506	0.001
	rs1256142	63980547	T/C	Intron 20		0.480	0.556	0.013
	rs2236222	63984935	T/C	Intron 21		0.086	0.068	0.322
	rs10137921	63985918	C/T	Exon 24	T761M	0.005	0.007	0.628
<i>MTHFD2</i> 2p13.1 16.7kb	rs11849530	63988165	A/G	Intron 24		0.234	0.247	0.610
	rs1256146	63990418	G/A	Intron 24		0.179	0.167	0.631
	rs34616731	63999040	T/A	Downstream 2.6 kb		0.178	0.189	0.663
	rs828858	74275702	T/A	Upstream 3.5kb		0.419	0.207	<0.0001
	rs6758506	74286460	G/A	Exon 2	Synonymous	0.002	0.004	0.740
	rs7587117	74302163	T/C	Downstream 6.2kb		0.334	0.165	<0.0001
	rs7571842	74314412	A/G	Downstream 18kb		0.447	0.605	<0.0001
	rs535107	11812055	A/G	Upstream 23kb		0.402	0.298	0.0005
	rs3737964	11789631	A/G	Upstream 0.94kb		0.244	0.183	0.018
	rs4846052	11780538	C/T	Intron 4		0.387	0.307	0.007
<i>MTHFR</i> 1p36.3 20kb	rs1801133	11778965	C/T	Exon 5	A222V(G677T)	0.315	0.394	0.009
	rs1801131	11777063	A/C	Exon 8	E429A(A1298C)	0.303	0.199	0.0002
	rs1476413	11774887	G/A	Intron 10		0.272	0.174	0.000
	rs2274976	11773514	G/A	Exon 12	R594Q	0.046	0.053	0.584
	rs1889292	11763530	G/A	Downstream 4.8kb		0.370	0.281	0.007
	rs2115540	77977363	T/C	Upstream 0.968kb		0.430	0.547	<0.0001
	rs2586179	77975061	C/A	Intron 1		0.496	0.530	0.263
	rs2562744	77961443	T/G	Intron 2		0.492	0.489	0.921
	rs7166109	77913530	C/T	Downstream 11kb		0.248	0.213	0.184
	rs12354209	235025875	A/G	Intron 1		0.378	0.341	0.120
<i>MTR</i> 1q43 105kb	rs4077829	235054413	G/T	Intron 9		0.404	0.401	0.901

Gene ^a	SNP	Chromosomal location (bp) ^b	Alleles ^c	Location ^b	Amino acid change	NHW MAP ^d	HCF [#]	p value
<i>MTOR</i> 5p15.3-p15.2 32kb	rs1806505	235063198	C/T	Intron 13		0.394	0.411	0.575
	rs6668344	235067949	C/T	Intron 14		0.411	0.413	0.966
	rs1770449	235104784	A/G	Intron 24		0.360	0.353	0.801
	rs1805087	235115123	A/G	Exon 26	D919G	0.247	0.243	0.887
	rs1266164	235117574	G/A	Intron 27		0.365	0.329	0.222
	rs11799647	235127544	A/G	Exon 33	I1259V	0.009	0.031	0.020
	rs1050993	235128928	G/A	3'UTR		0.358	0.337	0.487
	rs162029	7918527	G/A	Upstream 3.7kb		0.196	0.534	<0.0001
	rs2303080	7931424	T/A	Exon 5	S284T	0.030	0.087	<0.0001
	rs13166314	7955525	T/A	Downstream 1.3kb		0.359	0.680	<0.0001
<i>NOS3</i> 7q36 23.5kb	rs1800779	150320876	A/G	Intron 1		0.377	0.210	<0.0001
	rs1800780	150329812	A/G	Intron 12		0.449	0.576	0.001
	rs891512	150339022	G/A	Intron 13		0.222	0.122	<0.0001
	rs3918211	150341840	T/C	Exon 26	Synonymous	0.004	0.007	0.444
	rs2373929	150345745	G/A	Downstream 3.1kb		0.402	0.615	<0.0001
	rs3788205	45788806	C/T	Upstream 2.0kb		0.292	0.266	0.230
	rs4819130	45782727	T/C	Intron 1		0.464	0.459	0.863
	rs1051266	45782222	G/A	Exon 2	H27R [†]	0.426	0.444	0.564
	rs2330183	45777720	T/C	Intron 2		0.421	0.444	0.451
	rs3788190	45761386	G/A	Intron 5		0.434	0.457	0.459
<i>TYMS</i> 18p11.32 15.8kb	rs7278825	45760370	G/A	Exon 6	A469V [†]	0.019	0.029	0.428
	rs12483377	45755537	G/A	Downstream 3.5kb		0.065	0.058	0.647
	rs10483080	45750430	G/C	Downstream 8.6kb		0.131	0.213	0.0002
	rs2853741	647352	C/T	Upstream 0.299kb		0.294	0.309	0.500
	rs502396	649236	T/C	Intron 1		0.472	0.412	0.054
	rs1001761	652103	C/T	Intron 2		0.454	0.398	0.069
	rs11540152	652215	T/C	Exon 3	F117L	0.017	0.011	0.580
	rs11540153	659117	C/T	Exon 4	T167I	0.004	0.004	1.000
	rs2853532	660414	C/T	Intron 3		0.324	0.334	0.726
	rs495139	666008	C/G	Downstream 3.1kb		0.416	0.396	0.390

^a Gene name, chromosomal location, size of gene.

^b SNP data source: NCBI Map - genome build 36.3.

^c Most common allele listed first.

^d Non-Hispanic white minor allele frequency.

^e Hispanic corresponding frequency to non-Hispanic white minor allele.

[#] SNPs with call rate less than 95% were removed from further analyses.

[†] May be exonic or intronic depending on transcript used.

SNP, single-nucleotide polymorphism; NHW, non-Hispanic white group.

Results of Single SNP Association Analyses by Ethnicity

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$p < 0.05$ indicated in bold

Table 3**Results of 2-SNP Haplotype Transmission Analysis[†]**

Ethnicity	Gene	SNP1	SNP2	p value	Haplotype with altered transmission
Non-Hispanic White	<i>BHMTZ</i>	rs476620	rs626105	0.002	A,G ↑
	<i>NOS3</i>	rs1800779	rs2373929	0.002	A,G ↑
Hispanic	<i>BHMT</i>	13567754	rs3733890	0.006	C,A ↓; C, G ↑
	<i>NOS3</i>	rs1800779	rs2373929	0.002	A,A ↑
	<i>MTHFS</i>	rs7166109	rs2115540	0.0010	T,C ↓
	<i>TYMS</i>	rs11540152	rs495139	0.003	T,C ↑
	rs11540153	rs495139	0.003	T,C ↑ C,C ↑	

[†] Only p values 0.01 shown, ↑ = increased, ↓ = decreased.

Table 4Gene interactions between SNPs in different folate pathway genes[‡]

A. NHW				
Gene A	Marker A	Gene B	Marker B	P-value
BHMT	rs567754	TYMS	rs495139	0.006
BHMT2	rs476620	BHMT	rs585800	0.005
BHMT2	rs2253262	CBS	rs2851391	0.010
FOLR1	rs11235462	CBS	rs1801181	0.008
FOLR1	rs1540087	CBS	rs234783	0.009
FOLR1	rs11235462	MTHFD1	rs2236224	0.008
FOLR1	rs1540087	MTHFS	rs2586179	0.0004
FOLR1	rs1540087	SLC19A1	rs4819130	0.007
FOLR2	rs2298444	CBS	rs234714	0.001
FOLR2	rs2276048	CBS	rs234714	0.007
FOLR2	rs2298444	MTHFS	rs2115540	0.007
MTHFD1	rs1256142	CBS	rs12329790	0.005
MTHFD1	rs2236224	CBS	rs12329790	0.009
MTHFD1	rs34616731	SLC19A1	rs3788205	0.001
MTHFD1	rs1256146	SLC19A1	rs3788205	0.007
MTHFD2	rs7587117	MTHFS	rs2586179	0.002
MTHFD2	rs7587117	MTHFS	rs2562744	0.002
MTHFD2	rs7571842	MTHFS	rs2562744	0.003
MTHFD2	rs7571842	MTHFS	rs2586179	0.004
MTHFD2	rs7587117	MTRR	rs162029	0.006
MTHFD2	rs828858	MTRR	rs162029	0.010
MTHFR	rs1889292	SLC19A1	rs1051266	0.003
MTHFR	rs1889292	SLC19A1	rs3788205	0.009
MTHFR	rs1476413	SLC19A1	rs4819130	0.009
MTHFS	rs2586179	TYMS	rs2853741	0.007
MTRR	rs2303080	MTHFD1	rs34616731	0.002
MTRR	rs2303080	MTHFD1	rs1256146	0.009
NOS3	rs1800779	CBS	rs1051319	0.003
NOS3	rs1800779	FOLR2	rs2276048	0.007
NOS3	rs2373929	MTHFD1	rs1076991	0.002
NOS3	rs891512	MTHFS	rs2586179	0.007
NOS3	rs1800779	MTHFS	rs2562744	0.009
B. Hispanic				
Gene A	Marker A	Gene B	Marker b	p-value
BHMT	rs645112	MTHFD1	rs1256146	0.010

B. Hispanic				
Gene A	Marker A	Gene B	Marker b	p-value
BHMT2	rs626105	CBS	rs1051319	0.008
BHMT2	rs476620	CBS	rs1801181	0.010
BHMT2	rs2253262	MTHFD1	rs2236225	0.009
BHMT2	rs682985	SLC19A1	rs2297291	0.005
BHMT2	rs1422086	SLC19A1	rs2297291	0.009
CBS	rs234783	SLC19A1	rs4819130	0.008
CBS	rs2851391	SLC19A1	rs3788190	0.009
MTHFD1	rs2236222	SLC19A1	rs2297291	0.002
MTHFD1	rs1885031	SLC19A1	rs2297291	0.008
MTHFD1	rs1076991	TYMS	rs502396	0.004
MTHFD1	rs1076991	TYMS	rs2853532	0.010
MTHFR	rs1889292	BHMT2	rs2253262	0.008
MTHFR	rs4846052	CBS	rs2851391	0.005
MTHFR	rs3737964	CBS	rs234784	0.009
MTHFR	rs1476413	FOLR1	rs11235462	0.001
MTHFR	rs3737964	FOLR1	rs11235462	0.004
MTHFR	rs1889292	FOLR2	rs2276048	0.006
MTHFR	rs1476413	FOLR2	rs2276048	0.010
MTHFR	rs1476413	SLC19A1	rs10483080	0.006
MTHFR	rs1801131	SLC19A1	rs10483080	0.009
MTR	rs12354209	MTHFD2	rs7587117	0.001
MTR	rs1770449	MTHFD2	rs7587117	0.003
MTR	rs1266164	MTHFD2	rs7587117	0.004
MTR	rs1806505	MTHFD2	rs7587117	0.005
MTR	rs1266164	MTHFD2	rs828858	0.005
MTR	rs6668344	MTHFD2	rs828858	0.007
MTR	rs1770449	MTHFD2	rs828858	0.008
MTR	rs4077829	MTHFD2	rs828858	0.009
MTR	rs12354209	MTHFD2	rs828858	0.010
MTRR	rs162029	CBS	rs234783	0.003
NOS3	rs3918211	CBS	rs1051319	0.007
NOS3	rs2373929	SLC19A1	rs12483377	0.010
TYMS	rs1001761	CBS	rs234785	0.008

[‡] only p-values 0.01 shown, NHW = nonHispanic white group

Table 5

Smoking by Gene Interaction in non-Hispanic White Group*

Gene	SNP	Location	No. informative matings	<i>p</i> value
<i>FOLR1</i>	rs11235462	Downstream	53	0.036
<i>FOLR2</i>	rs651646	Intron 1	70	0.024
<i>FOLR2</i>	rs2298444	Intron 4	54	0.047
<i>FOLR2</i>	rs2276048	Downstream	93	0.012

* Additive model.

Table 6

Associated SNPs with Predicted Transcription Factor Binding Potential

Gene	SNP	Allele type	A libaba2	Program used to predict binding sites		TESS
				Patch		
<i>BHMT</i>	rs645112	Ancestral A	C/EBP6	STAT5A		MGF, NF-1
		Alternate C	HSF1	STAT6, STAT5A		C/EBPβ, IL-6, STAT6
<i>BHMT2</i>	rs22553262	Ancestral C	Sp1, NF-1	Sp1, ER-α, ER-β		Sp1, ER-α
		Alternate A	ER, USF	CAR, RAR-α1, RAR-beta, RXR-α, ER-α		AP-1, ER-α
	rs476620	Ancestral G	None	AREB6		None
		Alternate A	C/EBP	TGIF		AP-1, YY1, C/EBP-α
	rs626105	Ancestral A	NF-1	NF-1		NF-1, LEF-1, TCF-1
		Alternate G	NF-kappa	NF-1, LBP, MyoD, NF-1, NF-γ		None
<i>CBS</i>	rs234785	Ancestral C	None	None		None
		Alternate G	None	None		None
	Rs234784	Ancestral T	None	FXR, RXR-alpha		RAR-γ, AP-1
		Alternate C	None	AP-1		None
	rs234783	Ancestral T	None	None		None
		Alternate C	None	None		None
<i>FOLR2</i>	rs651646	Ancestral A	Sp-1	None		AP-2, AP-2α, AP-2αA, AP-2αB
		Alternate T	None	HAP2, HAP3, NF-YA		AP-2, AP-2α, AP-2αA, AP-2αB
<i>MTHFD1</i>	rs1076991	Ancestral A	AP-2α, NF-1, sp1, NF-kappaB	AP-2, AP-2α, AP-2αA, AP-2αB, NF-1, VDR, RXR-α		AP-2, AP-2α, AP-2αA, AP-2αB LBP-1
		Alternate G	YY1, AP-2	VDR, RXR-α		AP-2, AP-2α, AP-2αA, AP-2αB, LBP-1
<i>MTHFD2</i>	rs828858	Ancestral T	None	None		IPF-1, GR
		Alternate A	None	NF-Y		None
<i>MTHFR</i>	rs3737964	Ancestral G	None	VDR, Sp1, Sp2, Sp3, Sp4		None
		Alternate A	Sp1	VDR		None
<i>MTHFS</i>	rs2115540	Ancestral T	GR, ISGF-3	AREB6		None
		Alternate C	NF-EM5	None		None
	rs2586179	Ancestral C	Oct-1	None		None
		Alternate A	None	NKX3A, MIG1, CREB		None
<i>MTR</i>	rs12354209	Ancestral A	NF-1, C/EBPα	None		AP-2α

Only SNPs with $p < 0.01$ in regulatory regions shown.

AP-1 or 2 (transcription factor activator protein 1 or 2); AREB6 (A tpa1 regulatory element binding protein 6); CAR: constitutive androstane receptor; C/EBP: CCAAT/enhancer binding protein; CREB: cyclic AMP-responsive element binding protein); ER- α or β : estrogen receptor α or β ; FOXJ1; (Forkhead box protein J1); FXR: alias of NR1H4, nuclear receptor family protein; GATA1,2,3,4: GATA transcription factor family protein); GR (glucocorticoid receptor); HAP (Huntingtin-associated protein); HNF (histone nuclear factor); HNF-3 (hepatocyte nuclear factor 3); HOXA9: homeobox protein A9; HSF: heat shock factor; ICSBP (IRF8 aka interferon consensus sequences binding protein); IL-6; interleukin 6; IPF-1: insulin promoter factor 1; IRF (interferon regulatory factor); ISGF-3 (interferon stimulated gene factor 3); LBP (lipopolysaccharide-binding protein precursor); LEFT1 (lymphoid enhancer-binding factor 1); MGF: mast cell growth factor; MIG1 (mitogen-inducible gene 1); NF-EMBS (myoblast determination protein); NF-1 (nuclear factor 1); NF-kappa (lymphocyte specific nuclear factor); NF-kappa: nuclear factor of kappa light polypeptide gene enhancer; NF-Y: nuclear factor Y; NKX3A (homeobox protein NK-3 homolog); Oct (octamer-binding transcription factor); POU1F1a: pituitary specific transcription factor 1a; RAR: retinoic acid receptor; RXR: retinoid X receptor; SRF (serum response factor); Sp-1 (Sp1 transcription factor); STAT5A or 6: signal transducer and activator of transcription 5A or 6; TCF-1 (T-cell specific transcription factor 1, including TCF-1(P), TCF-1A, TCF-1B, TCF-1C, TCF-1E, TCF-1F, TCF-1G, TCF-2alpha); TGIF: 5'-TG-3' interacting factor; USF: upstream transcription factor 1; VDR: 1,25-dihydroxyvitamin D3 receptor; YAP1: yes-associated protein 1; YY-1 (transcriptional repressor protein YY1).