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Genetic Association Study of Putative Functional Single Nucleotide Polymorphisms of Genes in Folate Metabolism and Spina Bifida

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Abstract

OBJECTIVE—We tested putative functional single nucleotide polymorphisms (SNPs) in genes which regulate the folate/homocysteine metabolism pathway for their contribution to spina bifida (SB) susceptibility.

STUDY DESIGN—The study consisted of 610 unrelated simplex SB patient families. Genotypes of 46 SNPs located in the coding sequence or promoter region of 11 genes were investigated. Associations between transmission of alleles and SB in the offspring were examined using the reconstruction-combined transmission disequilibrium test.

RESULTS—Significant association of SNP rs5742905 in cystathionine- β -synthase (*CBS*), rs1643649 in dihydrofolate reductase (*DHFR*), rs2853533 in thymidylate synthetase (*TYMS*), and rs3737965 in methylene-tetrahydrofolate-reductase (*MTHFR*) was found ($p= 0.015, 0.041, 0.021,$ and 0.007 respectively).

CONCLUSION—Transmission disequilibrium of SNP alleles in *CBS*, *DHFR*, *MTHFR* and *TYMS* confers an increased susceptibility to SB.

Key words/phrases

spina bifida; single nucleotide polymorphism; folate/homocysteine metabolism genes

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Introduction

Neural tube defects (NTDs) are one of the most common birth defects in the United States, occurring in 2/10,000 births annually.¹ After a significant decline in the rates of spina bifida from 1995–1999, recent data demonstrates from 2003–2005, the rate of spina bifida has remained essentially unchanged.^{1, 2} Neurulation, the process of neural tube formation, is completed 28 days after conception.³ Disruption of neurulation which occurs during or prior to this time may result in a defect, or failure of closure of the neural tube. A variety of clinical abnormalities can result. The most common defects occur cranially (anencephaly) or caudally (spina bifida). Open NTDs are the most severe with anencephaly being lethal. Individuals affected with spina bifida (SB) who survive are at increased risk of early death and typically require extensive medical and surgical care.⁴

The etiology of most NTDs are multifactorial, or nonsyndromic, in origin resulting from both genetic and environmental influences. Studies have shown that in pregnancies affected by NTDs, the mothers have lower plasma levels of vitamin B₁₂ and folate than in those not affected.⁵ Maternal folate status is foremost in the prevention of occurrence and recurrence of many NTDs.^{6–8} Despite all of the positive results from epidemiologic studies and interventions regarding folate, the mechanisms underlying the relationship of folate status and NTD risk are not fully understood.^{9, 10}

Genetic background has been implicated as an important risk factor for NTDs. In addition, the incidence of NTDs varies geographically and can vary within ethnic and racial groups as well. In the United States Hispanics have one of the highest rates of NTDs.^{1, 4, 11} The genetic contribution of folate metabolism to NTDs has been investigated for two decades. The gene encoding the enzyme methylenetetrahydrofolate reductase (*MTHFR*) which catalyzes the reduction of 5, 10-methylenetetrahydrofolate (5, 10-MTHF) to 5-MTHF, a methyl group donor to convert homocysteine to methionine by methyltransferases, has been well studied. The most commonly studied variants in this gene are the polymorphisms C677T and A1298C. Both of these variants have been associated with increased risk of NTDs in various populations^{12–15} but the association is not universal.¹⁶ The *MTHFR* 667T variant has been shown to be more common in Hispanic individuals.^{4, 17} The enzyme activity of *MTHFR* with the 677T variant only produces approximately 50% of the wildtype 677C variant.¹⁸ This association of the *MTHFR* polymorphism with NTDs has led to inquires into other genes involved in folate transport and metabolism including thymidylate synthase (*TYMS*), dihydrofolate reductase (*DHFR*), and methylenetetrahydrofolate dehydrogenase (*MTHFD1*) genes that have also demonstrated varying associations to spina bifida.^{19–22}

Research into the genetic contribution of complex diseases that result from concomitant effects of environment, behavior, and genetic factors has been stimulated by the immense amount of data generated by the Human Genome Project. Research has revealed that while 99% of human DNA sequences are the same, variation also exists. There are different types of variation in DNA with the simplest and most frequent being single nucleotide polymorphisms (SNPs). SNPs are variations in the DNA sequence that occur when a single nucleotide (A, T, G or C) in the genome can be replaced by another nucleotide, with each nucleotide representing an allele of the SNP. In the vast majority of cases SNPs do not cause disease but such variants are stronger candidates than other genetic markers for locating disease susceptibility loci. SNPs occur approximately 1 in every 1000 base pairs of a sequence, with each individual having their own unique heritable pattern. SNPs may be linked, or inherited together as a consequence of their physical proximity on a single chromosome and may then be transmitted in groups, or linkage blocks, in a non-random fashion. In contrast, linkage disequilibrium (LD) is the nonrandom occurrence of specific SNP allele patterns at different loci. The distinction between

the two is that linkage is within a family association, while linkage disequilibrium is a population association. By comparing unique SNP patterns between two groups of individuals having different phenotypes (e.g. eye or hair color), geneticists can pinpoint the SNP patterns and genes associating with the phenotypes (i.e. genetic association). The aim in our study was to analyze the SNP patterns of parents and affected children with SB to determine how often these unique patterns were transmitted to affected offspring. The transmission disequilibrium test (TDT) measures association and linkage in families comparing the frequency of transmission of SNPs from parents to affected offspring. If there is no association with the disease the two alleles of a SNP have an equal chance of being transmitted, however if one allele of the SNP increases the risk of disease, it will be transmitted to the affected offspring more often than expected by chance alone.

The function and interaction of each of the 11 folate metabolism pathway genes are depicted in Figure 1. In addition to the genes directly coding for enzymes in the folate metabolism pathway shown in Figure 1, there are several other categories of genes involved more indirectly in folate metabolism. While cell proliferation and differentiation are important during neural tube folding, cell death/apoptosis also plays an important role during fusion of the neural roof plate and the surface ectoderm. Delay of the separation between the neural roof plate and surface ectoderm will delay the infiltration of mesenchymal cells between the two tissues layers required for neural arch formation. The MTHFR substrate, 5, 10-N-methylene-THF, donates a methyl group for the synthesis of deoxyribonucleotide dTMP catalyzed by TYMS with dihydrofolate as a byproduct. Interestingly, dihydrofolate and its precursors (dihydropterines) from other pathways are known to be toxic to animals.^{23, 24} Active cell proliferation during neural tube formation requires a sufficient supply of deoxyribonucleotides and produces a substantial amount of dihydrofolate through TYMS activity. This observation highlighted DHFR's activity in regulating dihydrofolate concentration within proliferating cells during neurogenesis and initiating apoptosis when suppressed.

Our objective was to test putative functional SNPs of 11 candidate genes known to regulate the folate/homocysteine metabolism pathway for their contribution to spina bifida susceptibility. The 11 candidate genes include methylene-tetrahydrofolate reductase (*MTHFR*), S-adenosylhomocysteine hydrolase (*AHCY*), betaine homocysteine methyltransferase (*BHMT*), cystathionine beta-synthase (*CBS*), dihydrofolate-reductase (*DHFR*), methionine adenosyltransferase 2A and 2B enzyme complex (*MAT2A*, *MAT2B*), methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*), 5-methyltetrahydrofolate homocysteine methyltransferase (*MTR*), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*), and thymidylate synthase (*TYMS*).

Materials and Methods

Study Population

In this study, patients affected with SB and their parents were enrolled from 1996 to 2006. Our study sample consisted of 610 cases comprised of either triads (affected individual and both parents) or duos (affected individual and one parent) recruited from Houston, Texas; Los Angeles, California; and Toronto, Canada. The study sample included 226 Caucasians, 334 Hispanics of Mexican descent, and 50 of other ethnic background. (Table 1) Level of the defect was determined by reviewing medical records and in some cases by review of X-rays. Sociodemographic, pregnancy history, maternal health history, and maternal exposures were obtained from the mothers of the affected cases. Vitamin supplementation was not elicited in the initial study but a survey to ascertain this information is currently ongoing. The project was approved by the institutional review board of The University of Texas Health Science Center at Houston.

SNP selection

We searched PubMed and public databases (<http://www.ncbi.nlm.nih.gov/SNP>, <http://genome.cse.ucsc.edu>, and <http://www.genecards.org/index.shtml>) for SNPs either shown to affect biological functions or predicted to affect biologic functions (transcription, splicing, translation, protein). A list of SNPs was submitted to Applied Biosystems Inc. (ABI, Foster City, California) for in silico analyses to exclude incompatible SNPs in the SNPlex multiplex reaction. Probes for a total of 48 compatible SNPs were made by ABI for SNPlex genotyping. (Table 2) Sample preparation

Blood samples and/or saliva samples were obtained from cases and both parents when possible. Genomic DNA from blood lymphocytes was extracted using the Puregene DNA Kit (Gentra Systems Inc, Minneapolis, MN) and DNA in saliva was extracted using the Oragene DNA collection kit (DNA Genotek, Inc., Ottawa, Ontario, Canada). Anonymous control DNA from 92 Hispanic individuals recruited from the Houston area and 92 Caucasian individuals from the Human Variation Panel-Caucasian Panel of 100 (HD100CAU) without a personal or family history of NTDs were used as negative controls and to evaluate the SNPs for Hardy Weinberg Equilibrium (HWE). No significant differences in the proportion of African and European Caucasian (CEU) genetic contributions between negative controls and patient samples were observed as reported in our previous study.¹⁷ Quality control of the genotyping was accomplished using known DNA samples from 30 Western European Caucasian families collected and used for building the SNP haplotypes map in humans (HapMap).

DNA Genotyping

SNP genotyping was carried out using the Applied Biosystems Inc. (ABI) SNPlex genotyping platform based on a multiplex oligonucleotide ligation/PCR/probe hybridization assay as described in our previous study.²⁵ Positive control DNA provided by ABI and no DNA controls were included for genotyping quality controls. The positive control DNA contains known genomic DNA to monitor the SNPlex platform for proper performance, while the no DNA control does not contain genomic DNA and will monitor background signals per experiments. Genotype data analyses were performed using the GeneMapper v4.0 software (ABI) and the genotypes called by the software were examined by at least two investigators before exporting and compiling for statistical analysis. Two SNPs were excluded from statistical analysis because they did not meet our criteria for having at least an 85 percent genotype call rate from GeneMapper.

Statistical Analysis

Genotypes of patients and parents were subjected to Mendelian error testing where SNPs with more than 10 families having errors were re-examined. SNPs were not analyzed when more than 10 families demonstrated non-Mendelian errors even after re-examination. In addition, patient families with more than 5 SNPs shown to have non-Mendelian errors were excluded from statistical analyses. Statistical analysis was performed with the model independent reconstruction combined-transmission disequilibrium test (RC-TDT) and standard TDT (S-TDT). RC-TDT is an analytic method for genetic association studies that utilizes affected offspring and is applicable when one of the two parents is missing as the primary data structure.^{26, 27} RC-TDT estimates the transmitted allele from the parental genotype based on observed genotypes and it is not subject to population stratification. Deviations from the expectation of allele frequencies under random transmission are considered evidence of linkage. All SNPs subjected to RC-TDT were also tested for HWE in NTD negative control samples to evaluate genotype calling errors and equilibrium of allele segregation. SNPs failing the HWE test were not analyzed. LD between SNPs in the same gene was examined using Haploview 4.1 (<http://www.broad.mit.edu/haploview/haploview>). RC-TDT results of SNP allele(s) with p

<0.05 were considered significant. Segregation of alleles for unlinked SNPs is expected to be a random process.

Results

To verify SNPlex genotyping platform accuracy, we compared genotypes we obtained for 45 CEU individuals used in the HapMap project with genotypes published in the SNP database. For all the SNPs successfully genotyped, 100% concordance was observed (data not shown). Duplicates of the positive controls used in separate SNPlex experiments produced reproducible genotypes.

Minor allele frequencies (MAF) were computed for the 46 successfully genotyped SNPs for controls, patients' and patients' parents according to their ethnicities and disease status. (Tables 3A and 3B) Frequencies of minor alleles of each SNP between control, patients and patients' parents appears to be quite similar within same ethnic group. The minor alleles and their frequencies among our Caucasian controls, patients and parents are consistent with those reported by the HapMap CEU. Minor allele and frequencies of the tested SNPs for Mexican American are not available in public databases so we could not carry out comparisons with the data we obtained from the Mexican American controls and patients we studied. Many of the 46 SNPs have minor allele frequency differences from 0.1–0.24 between Caucasian and Mexican Americans. Higher MAF for *BHMT* rs3733890, rs4703772; *MAT2A* rs1078004; *MTR* rs2229276; and *MTRR* rs326118, rs162036 and rs9332 were seen in Mexican Americans than Caucasian Americans. Lower MAF for *BHMT* rs585800; *DHFR* rs1643649; *MAT2B* rs17061795; *MTHFD1* rs8003379; *MTHFR* rs4846049; and *MTRR* rs1532268 were seen in Mexican Americans than Caucasian Americans.

LD analyses on Caucasian samples from HapMap did not suggest the SNPs we tested were linked and segregated together. However, some SNPs we tested were not included in the HapMap study, therefore we performed LD analyses using genotype data from the Caucasian and Hispanic controls in our study using Haploview 4.1. Three LD blocks between the 46 genotyped SNPs were identified with r^2 threshold set at 0.8: rs819133, rs819156 and rs819146 in *AHCY*; rs326118, rs162036 and rs9332 of *MTRR* and rs2287780 with rs16879334 in *MTRR* (data not shown). Alleles in the same block are expected to segregate together and show similar significance to disease association. The other SNPs tested are not linked.

The analysis of the entire data set for the 46 successfully genotyped SNPs in the 11 genes found significant preferential transmission of alleles in *CBS* rs5742905 (T allele, $p=0.015$), *DHFR* rs1643649 (T allele, $p=0.041$), *TYMS* rs2853533 (G allele, $p=0.021$), and *MTHFR* rs3737965 (C allele, $p=0.007$). (Table 4) All four SNPs in NTD negative controls were in HWE. (Table 3A & 3B) The remaining 42 SNPs did not show significant association by RC-TDT.

Comment

The study of folate and its association with NTDs is an ongoing endeavor that has led to countless studies of different genes involved in the folate metabolism pathway. We looked for a genetic association of putative functional SNPs between 11 candidate genes and susceptibility to a specific type of NTD, spina bifida. In our study we demonstrated parental transmission disequilibrium in four SNPs, on four different genes not previously reported, which confers an increased susceptibility to SB in their offspring.

The strengths of our study include a patient sample size significantly larger than prior studies and use of the family based RC-TDT method known to be independent of model and ethnicity. Our sample is comprised of approximately 60% Mexican Americans, a group known to have one of the highest incidences of SB. A total of 46 putative functional SNPs on 11 candidate

genes in folate/homocysteine metabolism pathways were studied. Forty two of the 46 SNPs examined did not reach significance in our study. There are several possible explanations for this negative finding. First, these 42 SNPs may not have a significant association with SB. Second, the study population size and power may not be sufficient to detect a genetic effect for these 42 SNPs, including the *BHMT* rs3733890, previously associated with increased risk of spina bifida in a family-based study consisting of 304 Caucasian American families.²⁸ Our study consisted of a total of 610 families; 226 are Caucasian American. We cannot discount that *BHMT* rs3733890 may be an ethnic specific risk factor that could be shown utilizing a larger sample size study with ethnically matched samples. Consistent with prior reports, our study did not find an association of *MTR* rs1805087 (p.D919G) and *MTRR* rs162036 (Lys350Arg) with risk for SB.²⁹

CBS

Many studies have found higher homocysteine in the plasma and amniotic fluid of mothers with NTD affected fetuses suggesting a failure to maintain homocysteine levels may be a contributing factor to NTD susceptibility.^{30–32} In our patient population the *CBS* SNP rs5742905, resulting in amino acid change from isoleucine to threonine, is significantly associated to risk of SB. *CBS* rs5742905 has not been examined previously in relation to SB. The most common *CBS* mutation studied is the 844ins68 allele, 11 bp downstream of rs5745905, which has shown varied association with NTDs or SB in different ethnic populations.^{28, 33, 34} Supporting this finding the *CBS* 844ins68 allele has been associated with lower serum homocysteine in a recent population study of over 10,000 subjects in Norway.³⁵

CBS rs5742905 Ile278Thr is associated with pyridoxine-responsive homocystinuria. The *CBS* 278Thr variant is less stable and has minimal *CBS* activity.³⁶ Further experiments are needed to determine if 278Thr contributes to risk of SB. Since the frequency of rs5742905 C allele is very low (0.01–0.02), we anticipate to further validate this finding with other linked SNPs in the *CBS* gene.

DHFR

In addition to converting dihydrofolate to tetrahydrofolate, *DHFR* also converts food folates into tetrahydrofolate. The *DHFR* rs1643649 has not been evaluated for its risk association to SB. Previous studies have identified a 19 base pair deletion polymorphism (19bpdel) that has shown to affect expression with conflicting results associated with SB risk in different populations.^{20, 22, 37, 38} We did not examine the 19bpdel because the SNPlex method is not designed to interrogate polymorphisms involving deletions. More studies will be need to evaluate the role of the 19bpdel to SB risk.

We were able to show increased susceptibility to SB in our study with *DHFR* rs1643649. The rs1643949 is in intron 3 of *DHFR* ~10kb downstream from the 19 bpdel. We found the *DHFR* rs1643649 allele C is predicted to lose pre-mRNA splicing factor binding which may lead to improper splicing. The significance of changes in splicing factor affinity to *DHFR* pre-mRNA splicing remains to be studied. Alternatively, rs1643649 may be linked to a SB risk allele yet to be identified.

MTHFR

MTHFR has been the major focus of research since the common SNPs C677T (rs1801133) and A1298C (rs1801131) were identified as one of the first genetic links to the NTD risk.^{12, 14–16, 39} The less frequent 677T variant of *MTHFR* has half of the enzyme activity of the wildtype enzyme and has been shown to be associated with low serum folate and high homocysteine.³⁵ A novel *MTHFR* polymorphism conferred increased risk for NTDs in a large Irish NTD cohort and was suggested to be physically linked to C677T.⁴⁰

MTHFR rs3737965 is located in the promoter sequence and therefore variants may affect transcriptional activity. The T allele variant of rs3737965 is predicted to constitute a progesterone receptor binding motif in contrast to the C allele. We examined rs3737965 and found that it was significantly associated with increased risk transmission in the offspring. We found both C677T and A1298C are not linked with rs3737965 ($r^2=0.02$ & 0.096 respectively). It is predicted the risk mechanism for rs3737965 is not due to simply linking with 677T or 1298C. Further experiments are needed to determine if the rs3737965 variant directly affects *MTHFR* transcription or is linked with a disease causing allele in our SB patients. Other studies have noted gene-gene interactions in various SNP alleles of *MTHFR*.^{28, 34, 41} All of these studies identified risk related to C677T. Two studies failed to show an associated gene-gene interaction but were limited by sample size.^{33, 42} Our findings demonstrate the importance to evaluate all SNPs throughout a candidate gene that are not linked to known risk alleles.

TYMS

The studies evaluating the 28 bp repeat polymorphism in the 5 prime untranslated region of TYMS association with SB remain inconclusive.^{43, 19} In our population, *TYMS* rs2853533 demonstrated an increased risk for SB. The MAF of rs2853533 is low (0.02–0.04); therefore validating this finding with other SNPs linked to rs2853533 is necessary.

In summary, to date no individual polymorphism in any candidate gene has been implicated as a risk factor in all studies. SNPs vary greatly in allele frequency among different geographic regions and ethnicities as we have demonstrated. Given the interconnectedness of the enzymes in the folate-homocysteine pathway we suspect that combinations of genetic variants in these enzymes may underlie NTD susceptibility. Our data contributes to the expanding body of literature.

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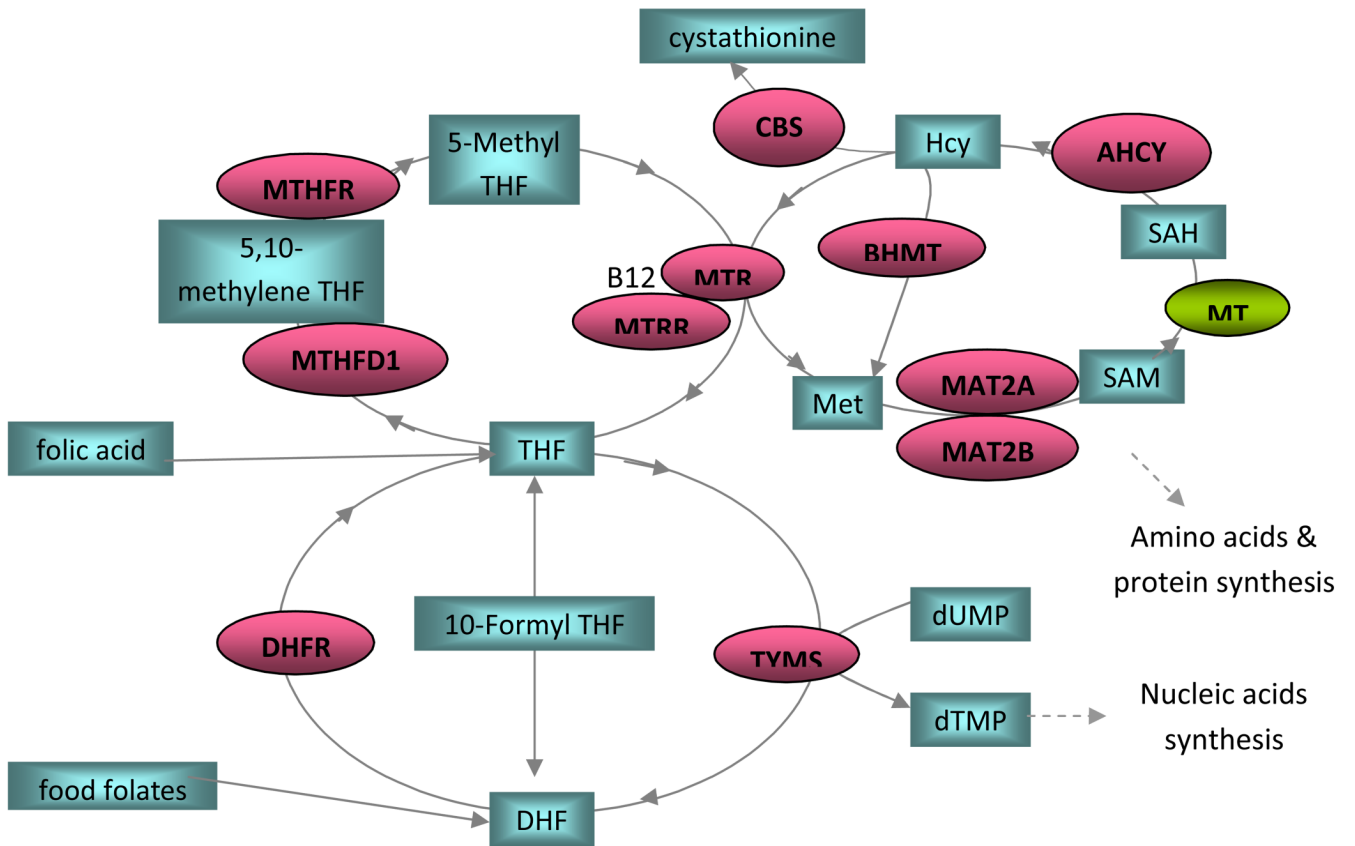


Figure 1. Folate Pathway in Humans

Substrates are shown in rectangular boxes. DHF: dihydrofolate; Hcy: homocysteine; MET: methionine; SAM: S-Adenosyl-methionine; SAH: S-Adenosylhomocysteine; THF: tetrahydrofolate

Enzymes are shown in ellipses. AHCY: S-adenosylhomocysteine hydrolase; BHMT: betaine-homocysteine methyltransferase; CBS: cystathionine-β-synthase; DHFR: dihydrofolate reductase; MAT2A: methionine adenosyltransferase II alpha; MAT2B: methionine adenosyltransferase II beta; MTHFD1: methylenetetrahydrofolate dehydrogenase; MTHFR: methylenetetrahydrofolate reductase; MTR: methyltetrahydrofolate-homocysteine methyltransferase; MTRR: MTR reductase; TYMS: thymidylate synthase; MT: methyltransferases

Table 1

Study Population Characteristics

Characteristic	Trios	Duos	Total
Race			
Caucasian	142	84	226
Mexican American	168	166	334
African American	4	14	18
Asian American	2	2	4
Other	7	5	12
Unknown	6	10	16
Gender			
Male	143	115	258
Female	150	123	273
Unknown	36	43	79

Total of 610 families, 329 trios, 281 duos are genotyped

Table 2

SNPs Genotyped

Gene Symbol	Gene Name	SNP ID	Chromosome	Putative Function
AHCY	S-adenosylhomocysteine hydrolase	rs819146 rs819156 rs7271501 rs819133	20	Promoter Intron 3 Intron 6 Intron 9
BHMT	betaine-homocysteine methyltransferase	rs16876512 rs3733890 rs4703772 rs585800	5	Promoter R239Q, ESE L264, ESE 3'-UTR, ESE
CBS	cystathionine-b-synthase	rs1788484 rs5742905 rs12613 rs706209	21	Promoter I278T, ESE 3'-UTR, ESE 3'-UTR, ESE
DHFR	dihydrofolate reductase	rs408626 rs1677658 rs1643649	5	Promoter Promoter Intron
MAT2A	methionine adenosyltransferase II alpha	rs1078004 rs2043675	2	R264, ESE Intron 7
MAT2B	methionine adenosyltransferase II beta	rs4869087 rs12515448 rs17061795 rs6898075		Intron 1 Intron 2 L175, ESE Intron 4
MTHFD1	methylene-tetrahydrofolate dehydrogenase	rs8003379 rs1950902 rs8016556 rs11627387 rs2281603	14	Intron 2 K134R Intron 16 Intron 26 Intron 27
MTHFR	methylene-tetrahydrofolate reductase	rs3737965 rs2066470 rs9651118 rs2066462 rs2274976 rs4846049	1	Promoter P39, ESE Intron 2 S352, ESE R594Q, ESE 3'-UTR
MTR	methyltetrahydrofolate-homocysteine methyltransferase	rs1805087 rs2229276 rs1131449 rs11799670	1	D919G, ESE A1048, ESE L1192, ESE 3'-UTR, ESE
MTRR	methyltetrahydrofolate-homocysteine methyltransferase reductase	rs326118 rs1532268 rs10064631 rs162036 rs2287780 rs16879334 rs9282787 rs9282788 rs9332	5	Promoter S175L L333V, ESE K350R, TRP R415C P450R, ESE 3'-UTR ESE 3'-UTR ESE 3'-UTR ESE
TYMS	thymidylate synthase	rs2853533 rs699517 rs2790	18	Intron 1 3'-UTR ESE 3'-UTR ESE

SNP: single nucleotide polymorphism, ESE: exonic splicing element, UTR: mRNA untranslated region, TRP: triple helix regulatory site, rs: reference SNP

Table 3

A Frequencies of minor alleles for Caucasian American samples

gene	SNP ID	Allele 1	Allele 2	minor allele	Caucasian American MAF				
					patient (N=230)	father (N=153)	mother (N=218)	parent (N=371)	controls (N=145)
AHCY	rs819133	G	T	T	0.16	0.09	0.19	0.14	0.13
	rs7271501	G	C	C	0.01	0.01	0.01	0.01	0.01
	rs819156	C	A	A	0.13	0.06	0.14	0.11	0.11
BHMT	rs819146	G	T	G	0.13	0.07	0.14	0.11	0.11
	rs16876512	C	T	T	0.13	0.11	0.11	0.11	0.11
	rs3733890	G	A	A	0.30	0.26	0.28	0.28	0.26
	rs4703772	C	T	T	0.01	0.01	0.03	0.02	0.02
	rs585800	A	T	T	0.26	0.28	0.26	0.27	0.26
CBS	rs706209	C	T	T	0.47	0.46	0.44	0.45	0.44
	rs12613	G	A	A	0.13	0.12	0.13	0.13	0.12
	rs742905	T	C	C	0.01	0.02	0.01	0.01	0.00
	rs1788484	C	T	T	0.39	0.38	0.36	0.37	0.40
DHFR	rs1643649	T	C	C	0.27	0.27	0.25	0.26	0.27
	rs1677658	G	T	T	na	na	Na	na	na
	rs408626	A	G	G	0.42	0.40	0.42	0.41	0.43
MAT2A	rs1078004	C	G	G	0.48	0.50	0.49	0.50	0.45
	rs2043675	T	C	C	0.34	0.28	0.37	0.33	0.32
MAT2B	rs4869087	A	C	C	0.25	0.30	0.28	0.29	0.31
	rs12515448	A	G	G	0.48	0.50	0.49	0.50	0.48
	rs17061795	A	G	T	0.13	0.17	0.15	0.16	0.17
	rs6898075	C	T	G	0.40	0.45	0.43	0.43	0.39
MTHFD1	rs8003379	A	C	C	0.23	0.20	0.20	0.20	0.23
	rs1950902	C	T	T	0.33	0.32	0.38	0.36	0.20
	rs8016556	T	C	C	0.32	0.31	0.35	0.33	0.35
	rs11627387	G	A	A	0.37	0.35	0.40	0.38	0.43
	rs2281603	A	G	G	0.26	0.22	0.26	0.24	0.26
MTHFR	rs4846049	G	T	T	0.29	0.28	0.32	0.31	0.35
	rs2274976	G	A	A	0.04	0.06	0.04	0.04	0.06
	rs2066462	C	T	T	0.05	0.04	0.04	0.04	0.06
	rs9651118	T	C	C	0.21	0.23	0.21	0.21	0.23

Caucasian American MAF

controls (N=145)

parent (N=371)

mother (N=218)

father (N=153)

patient (N=230)

minor allele

Allele 2

Allele 1

SNP ID

gene

MTR	rs2066470	C	T	0.11	0.08	0.11	0.10	0.09
	rs3737965	C	T	0.06	0.08	0.06	0.07	0.06
	rs1805087	A	G	0.19	0.20	0.16	0.18	0.19
	rs2229276	A	G	0.47	0.46	0.47	0.47	0.39
	rs1131449	T	C	0.39	0.38	0.41	0.40	0.44
MTRR	rs11799670	A	G	0.03	0.03	0.02	0.02	0.02
	rs326118	T	G	0.12	0.09	0.12	0.11	0.14
	rs1532268	T	A	0.40	0.39	0.40	0.39	0.37
	rs10064631	G	C	na	na	Na	na	na
	rs162036	A	G	0.12	0.10	0.12	0.11	0.14
	rs2287780	C	T	0.06	0.07	0.05	0.06	0.04
	rs16879334	C	G	0.05	0.05	0.05	0.05	0.03
	rs9282787	T	C	0.24	0.21	0.24	0.23	0.18
	rs9282788	T	C	0.08	0.07	0.06	0.07	0.03
	rs9332	C	T	0.13	0.11	0.13	0.13	0.14
TYMS	rs2853533	G	C	0.02	0.04	0.02	0.02	0.04
	rs699517	C	T	0.30	0.28	0.30	0.29	0.36
	rs2790	A	G	0.19	0.16	0.21	0.19	0.24

Table 3B Frequencies of minor alleles for Mexican American samples

gene	SNP ID	Allele 1	Allele 2	minor allele	Mexican American MAF				
					patient (N=336)	father (N=179)	mother (N=321)	parent (N=500)	controls (N=92)
AHCY	rs819133	G	T	T	0.16	0.12	0.17	0.16	0.16
	rs7271501	G	C	C	0.01	0.01	0.02	0.02	0.06
	rs819156	C	A	A	0.11	0.10	0.11	0.10	0.11
	rs819146	G	T	G	0.13	0.11	0.14	0.13	0.14
	rs16876512	C	T	T	0.10	0.10	0.11	0.11	0.05
BHMT	rs3733890	G	A	A	0.36	0.37	0.40	0.39	0.41
	rs4703772	C	T	T	0.21	0.18	0.21	0.20	0.20
	rs585800	A	T	T	0.15	0.13	0.16	0.15	0.14
	rs706209	C	T	T	0.32	0.30	0.31	0.30	0.38
	rs12613	G	A	A	0.15	0.15	0.15	0.15	0.09
DHFH	rs5742905	T	C	C	0.01	0.01	0.02	0.01	0.02
	rs1788484	C	T	T	0.31	0.29	0.32	0.31	0.31
	rs1643649	T	C	C	0.20	0.18	0.21	0.20	0.12
	rs1677658	G	T	T	na	na	na	na	na
	rs408626	A	G	G	0.56	0.55	0.54	0.54	0.50
MAT2A	rs1078004	C	G	G	0.59	0.62	0.63	0.62	0.57
	rs2043675	T	C	C	0.29	0.29	0.28	0.29	0.30
	rs4869087	A	C	C	0.28	0.24	0.28	0.27	0.34
	rs12515448	A	G	G	0.47	0.43	0.46	0.45	0.42
	rs17061795	A	G	G	0.11	0.10	0.11	0.10	0.07
MTHFD1	rs6898075	C	T	T	0.40	0.38	0.39	0.39	0.33
	rs8003379	A	C	C	0.17	0.22	0.12	0.16	0.10
	rs1950902	C	T	T	0.28	0.23	0.33	0.29	0.17
	rs8016556	T	C	C	0.28	0.26	0.25	0.26	0.28
	rs11627387	G	A	A	0.37	0.34	0.33	0.33	0.44
MTHFR	rs2281603	A	G	G	0.26	0.25	0.27	0.26	0.30
	rs4846049	G	T	T	0.20	0.23	0.19	0.21	0.14
	rs2274976	G	A	A	0.03	0.04	0.05	0.05	0.04
	rs2066462	C	T	T	0.02	0.02	0.02	0.02	0.03
	rs9651118	T	C	C	0.24	0.25	0.26	0.26	0.30
MTR	rs2066470	C	T	T	0.11	0.10	0.11	0.11	0.06
	rs3737965	C	T	T	0.08	0.07	0.10	0.09	0.07
	rs1805087	A	G	G	0.23	0.19	0.24	0.22	0.16

Table 3B Frequencies of minor alleles for Mexican American samples

gene	SNP ID	Allele 1	Allele 2	minor allele	Mexican American MAF				
					patient (N=336)	father (N=179)	mother (N=321)	parent (N=500)	controls (N=92)
MTRR	rs229276	A	G	G	0.47	0.46	0.47	0.47	0.49
	rs1131449	T	CT	C	0.38	0.40	0.38	0.39	0.45
	rs11799670	A	G	G	0.10	0.10	0.11	0.10	0.09
	rs326118	T	G	G	0.38	0.32	0.39	0.36	0.33
	rs1532268	G	A	A	0.22	0.25	0.19	0.21	0.19
	rs10064631	G	C	C	na	na	na	na	na
	rs162036	A	G	G	0.41	0.36	0.43	0.40	0.38
	rs2287780	C	T	T	0.14	0.13	0.15	0.14	0.13
	rs16879334	C	G	G	0.12	0.12	0.14	0.13	0.11
	rs9282787	T	C	C	0.10	0.10	0.10	0.10	0.09
TYMS	rs9282788	T	C	C	0.11	0.08	0.11	0.10	0.05
	rs9332	C	T	T	0.41	0.36	0.40	0.39	0.33
	rs2853533	G	C	C	0.02	0.03	0.02	0.02	0.01
	rs699517	C	T	T	0.35	0.34	0.33	0.34	0.44
	rs2790	A	G	G	0.24	0.24	0.24	0.24	0.32

MAF = minor allele frequency, na = not available due to low % genotyping efficiency, rs=reference SNP

MAF = minor allele frequency, na = not available due to low % genotyping efficiency, rs=reference SNP

Table 4

RC-TDT Results

Gene Symbol	dbSNP ID	Allele	RC-TDT p-value
CBS	rs5742905	T	0.0156
DHFR	rs1643649 *	T	0.0411
MTHFR	rs3737965	C	0.0076
TYMS	rs2853533	G	0.0213

* Note: rs11951883 merged with rs1643649. rs=reference SNP