Genetic Epidemiology 33:247–255 (2009)

Oral Facial Clefts and Gene Polymorphisms in Metabolism of Folate/One-Carbon and Vitamin A: A Pathway-Wide Association Study

Abee L. Boyles,[1]* Allen J. Wilcox,[1] Jack A. Taylor,[1] Min Shi,[2] Clarice R. Weinberg,[2] Klaus Meyer[3] Åse Fredriksen,[3] Per Magne Ueland,[3] Anne Marte W. Johansen,[4] Christian A. Drevon,[4] Astanand Jugessur,[5] Truc Nguyen Trung,[6] Håkon K. Gjessing,[6] Stein Emil Vollset,[6] Jeffrey C. Murray,[7] Kaare Christensen[8] and Rolv T. Lie[6]

[1]*Epidemiology Branch, NIEHS/NIH, Durham, North Carolina*
[2]*Biostatistics Branch, NIEHS/NIH, Durham, North Carolina*
[3]*Department of Pharmacology, University of Bergen, Bergen, Norway*
[4]*Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, Oslo, Norway*
[5]*Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Melbourne, Australia*
[6]*Department of Public Health and Primary Health Care, University of Bergen, Bergen, Norway*
[7]*Department of Pediatrics, University of Iowa, Iowa City, Iowa*
[8]*Center for Prevention of Congenital Malformation, Epidemiology Unit, University of Southern Denmark, Odense, Denmark*

An increased risk of facial clefts has been observed among mothers with lower intake of folic acid or vitamin A around conception. We hypothesized that the risk of clefts may be further moderated by genes involved in metabolizing folate or vitamin A. We included 425 case-parent triads in which the child had either cleft lip with or without cleft palate (CL/P) or cleft palate only (CPO), and no other major defects. We analyzed 108 SNPs and one insertion in 29 genes involved in folate/one-carbon metabolism and 68 SNPs from 16 genes involved in vitamin A metabolism. Using the Triad Multi-Marker (TRIMM) approach we performed SNP, gene, chromosomal region, and pathway-wide association tests of child or maternal genetic effects for both CL/P and CPO. We stratified these analyses on maternal intake of folic acid or vitamin A during the periconceptional period.

As expected with this high number of statistical tests, there were many associations with P-values < 0.05; although there were fewer than predicted by chance alone. The strongest association in our data (between fetal *FOLH1* and CPO, $P = 0.0008$) is not in agreement with epidemiologic evidence that folic acid reduces the risk of CL/P in these data, not CPO. Despite strong evidence for genetic causes of oral facial clefts and the protective effects of maternal vitamins, we found no convincing indication that polymorphisms in these vitamin metabolism genes play an etiologic role. *Genet. Epidemiol.* 33:247–255, 2009. © 2008 Wiley Liss, Inc.

Key words: cleft lip; cleft palate; dietary supplements; folic acid; genetics; metabolism; vitamin A

Additional supporting information may be found in the online version of this article.
Contract grant sponsor: National Institutes of Health; Contract grant number: DE085592, RO1 DE-11948-04, N01-HG-65403, P50 DE-16215, R37 DE-0559; Contract grant sponsor: National Institute of Environmental Health Sciences; Contract grant number: Z01 ES049027-11, Z01 ES040007; Contract grant sponsor: the Research Council of Norway; Contract grant number: 166026/V50; Contract grant sponsor: Foundation to promote research into functional vitamin B12-deficiency; Contract grant sponsor: the Freia Foundation; Contract grant sponsor: the Throne-Holst Foundation; Contract grant sponsor: Faculty of Medicine, University of Oslo.
*Correspondence to: Abee L. Boyles, Epidemiology Branch, Mail Drop A3-05, P.O. Box 12233, National Institute of Environmental Health Sciences/NIH, Durham, NC 27709. E-mail: boylesa@niehs.nih.gov
Received 29 August 2008; Accepted 5 September 2008
Published online 1 December 2008 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/gepi.20376

INTRODUCTION

Oral facial clefts, including cleft lip and palate, have a strong genetic basis as determined by many studies of recurrence risks in relatives and by segregation analyses in diverse populations [Clementi et al., 1997; Murray et al., 1997; Palomino et al., 1997; Scapoli et al., 1999; Sivertsen et al., 2008; Vieira et al., 2003]. Supplemental intake of folic acid and multivitamins around conception is suggested to provide protection from these birth defects [Badovinac et al., 2007; Bille et al., 2007; Chevrier et al., 2007; Krapels et al., 2004; Mitchell et al., 2003; van Rooij et al., 2004]. In the Norwegian population studied here, folic acid supplementation provided a 39% reduction in the risk of cleft lip with or without cleft palate (CL/P) [Wilcox et al., 2007], whereas increased total vitamin A intake from food and supplements reduced the risk of cleft palate only (CPO) by 53% [Johansen et al., 2008]. Excessive vitamin A can be teratogenic, but not at the levels observed in this population [Soprano and Soprano, 1995]. However, it is

© 2008 Wiley-Liss, Inc.

unclear to what extent the genetic risk of clefts is intertwined with the risk from vitamin deficiencies despite numerous smaller-scale association studies showing modest effects, including our own [Boyles et al., 2008; Jugessur et al., 2003a,b,c].

MATERIAL AND METHODS

PARTICIPANTS

Families of patients with oral facial clefts born in Norway between 1996 and 2001 were enrolled in the study. The overall study design has been described previously [Wilcox et al., 2007]. Of approximately 300,000 live births during this time, 676 were referred for corrective surgery on oral facial clefts. Twenty-four babies were excluded due to death or mothers who did not speak Norwegian. Five hundred and seventy-three of the remaining 652 families (88%) agreed to participate in the study. Of 1,022 randomly sampled live births, 763 (75%) were enrolled using the same exclusion criteria. All parents provided informed consent. For this study only isolated cases of oral facial clefts were analyzed. Cases were excluded if another birth defect was reported on the mother's questionnaire, in the Medical Birth Registry, or in medical records from the time of surgery [Nguyen et al., 2007]. We used controls only for assessing deviations from Hardy-Weinberg equilibrium (HWE) and for calculations of linkage disequilibrium (LD). Table I shows the number of case families stratified by cleft type, the number of families of cases with an isolated cleft on whom we had information on both genotype and vitamin intake, and who represent the analyses presented here [Wilcox et al., 2007].

GENOTYPING

Most of the SNPs in this study comprise a subset of those assayed as part of a larger project exploring candidate genes and clefts in Norwegian and Danish populations. Genes potentially implicated in oral facial clefts were chosen from published association and linkage studies, human cytogenetic rearrangements, Mendelian forms of clefting identified in the OMIM™ database (Baltimore, MD; www.ncbi.nlm.nih.gov/omim), gene knockout experiments in mice, and gene expression studies in human and mouse embryonic tissues [Brown et al., 2003; Cai et al., 2005; Gong et al., 2005; Jugessur and Murray, 2005; Lidral and Moreno, 2005; Mukhopadhyay et al., 2004]. A list of 357 candidate genes was generated which included functional categories likely related to oral facial cleft etiology such as growth factors, detoxification genes, genes for syndromes that include clefts, and vitamin metabolism-related genes.

SNPs in these genes were selected primarily using CEPH data from the International HapMap Consortium (www.hapmap.org) to evaluate their haplotype-tagging properties, minor allele frequency (MAF), and gene coverage. Supplemental SNPs were chosen using dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/), JSNP (snp.ims.u-tokyo.ac.jp), genome bioinformatics at UCSC (genome.ucsc.edu), CHIP SNPper tool (snpper.chip.org), and SeattleSNPs (pga.mbt.washington.edu). The SNPs were prioritized based on prior evidence of an association with clefting, coding SNPs, and an MAF of at least 10%. Additional selected SNPs were intragenic, in putative regulatory regions in the UTRs, or had haplotype-tagging properties. SNPs were evaluated using HAPLOVIEW version 2.05 (www.broad.mit.edu/mpg/haploview/index.php) [Barrett et al., 2005], BEST (www.genomethods.org/best/index.htm) [Sebastiani et al., 2003], and SNP Browser™ (Applied Biosystems, Foster City, CA) to determine LD patterns and haplotype block structures for the selection of haplotype tagging SNPs.

Some SNPs were removed due to properties detrimental to assay design: nearby palindromic sequences, GC- and AT-rich regions, repetitive sequences, and sequences that are similar to other human sequences. SNP assays designed by Illumina were evaluated by a "design score" that tests each SNP's performance on the GoldenGate™ (San Diego, CA; www.illumina.com) platform. After thorough evaluation a custom panel of 1,536 SNPs was designed for the 357 genes and genotyped by the US Center for Inherited Disease Research (CIDR, www.cidr.jhmi.edu).

SNPs with call rates below 95% were eliminated, as were those out of HWE ($\chi^2 > 10$) in the control population as determined by an exact test [Wigginton et al., 2005]. In addition, eight families were removed for excessive Mendelian inconsistencies (65 and more, while those included had no more than eight inconsistencies and there were no families with between nine and 64 inconsistencies). Those with high numbers of Mendelian inconsistencies were probably the result of sample switches or misidentified paternity. Many of these same family inconsistencies have been previously reported [Boyles et al., 2008].) SNPs on the X and Y chromosomes were used to confirm the sex of subjects and their parents.

The intent of the overall candidate gene study was to evaluate a large number of SNPs in genes thought to be related to oral facial clefts in the Norwegian and Danish populations. These data also allow more specific exploration of gene-environment interaction for exposures associated in our data with the risk of clefting. The Norway study included detailed questionnaires of maternal exposures that were not available for the Danish samples. Our previous work in the Norwegian population showed a reduction in the risk of clefts from folic acid supplementation [Wilcox et al., 2007], vitamin A intake [Johansen et al., 2008], and an SNP in *CBS*, a one-carbon metabolism gene [Boyles et al., 2008].

TABLE I. Samples sizes for study participants subdivided by cleft type, those genotyped, and isolated case families analyzed by each stratum of maternal vitamin intake

	CL/P	CPO	All clefts
Participating families	**377**	**196**	**573**
Genotyped participants	**374**	**188**	**562**
Isolated with folic acid information	**311**	**114**	**425**
$<400\,\mu g$	272	93	365
$400^{+}\,\mu g$	39	21	60
Isolated with Vitamin A information	**285**	**112**	**397**
$<1257\,\mu g$	151	74	225
$1257^{+}\,\mu g$	134	38	172

From the CIDR genotyping set, 114 SNPs were chosen in 27 genes involved with folate/one-carbon metabolism. Nine of these SNPs had poor call quality and three were not in HWE, leaving 102 SNPs for analysis. An additional seven polymorphisms in five folate/one-carbon metabolizing genes had been previously genotyped in these samples by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [Boyles et al., 2008; Meyer et al., 2004], and are included in this analysis. Figure 1 diagrams the relative genomic positions of the 29 folate/one-carbon metabolism genes and functional type of the 109 genotyped polymorphisms. Further description of these genes and their functional categories are included in Supplemental Table I. Sixteen genes related to vitamin A metabolism are also included in Figure 1. This set of genes includes transforming growth factor signaling genes which are part of other signaling pathways but have been linked to vitamin A metabolism as well [Abbott et al., 2005; Baroni et al., 2006]. These genes are described further in Supplemental Table II. Sixty-eight of 73 selected vitamin A-related SNPs were analyzed (four had poor call rates and one was not in HWE).

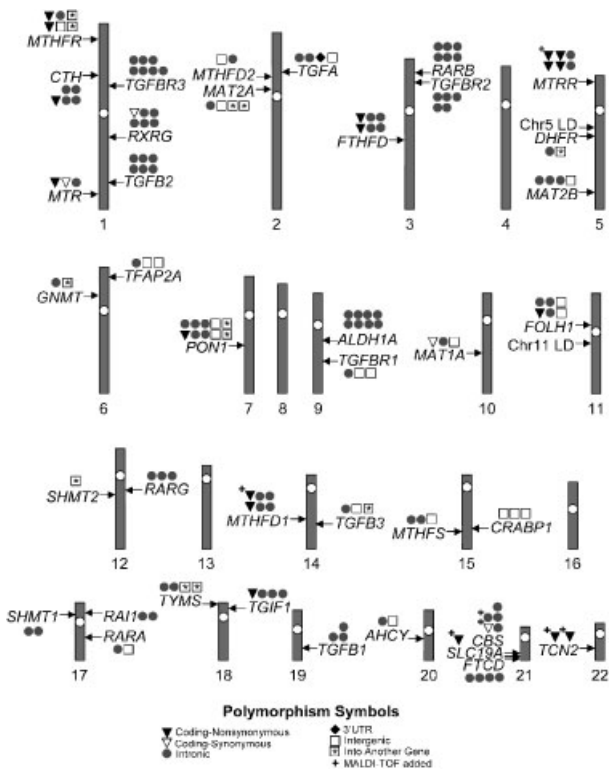

Fig. 1. Genomic location of genes in the folate/one-carbon (left of the chromosome) and the vitamin A (right of the chromosome) pathways. SNPs indicated by a square with a star were considered in the region of the gene of interest, but actually fell within a neighboring gene. Those indicated with a + were obtained from MALDI-TOF genotyping performed prior to the CIDR genotyping, and previously reported [Boyles et al., 2008]. SNPs in the 5q and 11q LD regions are detailed in Figure 2. 172 × 214 mm (300 × 300 DPI)

VITAMIN SUPPLEMENTATION

Mothers were mailed questionnaires approximately 3 months after delivery regarding vitamin intake during the 6 months prior to and the first 3 months of pregnancy (www.niehs.nih.gov/research/atniehs/labs/epi/studies/ncl/question.cfm). If mothers reported taking a vitamin supplement, they were asked for the product name. Mothers mailed empty pill bottles or product labels to the study office for verification of the product and dosage.

The facial structures of the embryonic lip and palate fuse during the first 2 months of gestation, so we considered mothers to be supplemented if they took folic acid for at least 1 month during the 3-month window starting a month before the last menstrual period and going through the first 2 months of pregnancy. Intake of 400 μg or more of folic acid supplements reduced the risk of CL/P, whereas no protective effect was seen in CPO and there was no protective effect at lower doses [Wilcox et al., 2007]. For the folate/one-carbon metabolism analysis, we therefore divided women into strata of <400 μg/day and over 400^{+} μg/day of folic acid from dietary supplements.

Total vitamin A from food and supplements was shown in this sample to reduce the risk of CPO in a dose-dependent manner, but not CL/P [Johansen et al., 2008]. Recommended levels of vitamin A during pregnancy vary among countries, and excessive vitamin A is teratogenic [Finnell et al., 2004; Soprano and Soprano, 1995]. Vitamin A level was determined from a food frequency questionnaire covering maternal diet and supplement use during the first 3 months of pregnancy as described previously [Johansen et al., 2008]. We divided the data set at the mean level of vitamin A (1,257 μg from both diet and supplements calculated as retinol plus 1/12 beta-carotene). The intake levels of both vitamins were used to stratify the data, but no formal test of gene-vitamin interaction was performed.

STATISTICAL ANALYSES

The Triad Multi-Marker test (TRIMM) allows assessment of associations with both the child's and mother's genes using multiple markers from mother-father-child triad families [Shi et al., 2007]. To assess a possible effect of inherited haplotypes, the genotype vector of the offspring is contrasted with that of a hypothetical ''complement'' child who would have inherited parental alleles not transmitted to the observed offspring. The difference between these two offspring genotype vectors has an expected value of 0 at each locus under the null hypothesis, similar to the pedigree disequilibrium test [Martin et al., 2000]. The TRIMM method allows for multiple linked SNPs, but does not assume that there is no recombination from parent to child. Permutation of the labels for case-versus-complement status (i.e. randomly multiplying the difference vector by +1 or −1) is used to evaluate statistical significance. To optimize over scenarios where risk depends on a single SNP and also over scenarios where risk instead depends on a multi-SNP susceptibility haplotype, TRIMM performs both maximum and Hotelling's T^2 tests, and generates a combined *P*-value from the two tests (sum_logP). The program also nominates risk-haplotype-tagging alleles, but does not need to either calculate or impute phase from the observed data.

The TRIMM test for a maternally mediated genetic effect is similar except the difference vector used is based on the difference between maternal and paternal genotypes. Under genetic mating symmetry the father serves as a genetic control for the mother of the child with cleft. When calculating the child genetic effect, the calculations assumed parental symmetry unless there was evidence of a maternal effect ($P < 0.05$), in which case families with missing genotypes are omitted from the analysis.

All genes were analyzed separately, generating a sum_logP gene score as well as max Z^2 scores for individual SNPs and a possible risk-haplotype was nominated if the overall max Z^2 P-value was less than 0.1. LD between all pairs of polymorphisms was calculated from the control population samples using Haploview version 4.0 [Barrett et al., 2005]. Among the folate/one-carbon metabolizing genes, we jointly analyzed three genes on chromosome 5 and three more on chromosome 11 due to high LD ($D' > 0.5$ and LOD > 2) between them. The LOD score is a measure of confidence in D'. An overall pathway analysis was also conducted for folate/one-carbon and for vitamin A.

RESULTS

When genes are in close proximity, LD can create dependency between results of individual genes. We therefore calculated LD between SNPs in the same pathway to identify regions in high LD for joint analysis. For the folate/one-carbon metabolizing genes on 5q11-14 there was significant LD among *DMGDH*, *BHMT2*, and *BHMT1* but not with *DHFR* (Fig. 2). *DMGDH*, *BHMT2*, and *BHMT1* were included in the Chr5 LD region and *DHFR* was analyzed separately. The folate/one-carbon metabolizing genes on 11q (FOLR1, FOLR2, and FOLR3) showed high LD with one another (Fig. 2), and so were analyzed jointly as the Chr11 LD region. All other folate/one-carbon and vitamin A metabolizing genes were not in LD with one another ($D' < 0.5$ or LOD < 2) and were analyzed separately.

The TRIMM sum_logP value was calculated for each gene or LD region, as were the max Z^2 scores for each individual SNP. For both the folate/one-carbon and vitamin A pathways, we found fewer tests with P-values < 0.05 than we would expect by chance. In the folate/one-carbon pathway we analyzed 25 genes or LD regions, two genetic effects (analyzing either for the child's or the mother's genotype), two types of facial clefts (CL/P or CPO), and two strata of vitamin intake. If these tests had all been independent then under the global null that all variants on these pathways are unrelated to clefting we would have expected ten of these 200 tests to produce P-values less than 0.05, but we observed only eight. Similarly with the 16 genes of the vitamin A pathway, we expected six of the 128 tests to have P-values less than 0.05, but we observed only four.

We graphically compared the observed P-values of the resulting 328 independent tests to an expected uniform distribution of P-values stratified by type of cleft and pathway (Fig. 3). This quantile-quantile plot compares the percentile of tests expected by chance to the observed P-values. Specific SNPs or LD regions with P-values < 0.05 have been labeled. In three of the four panels, there is very

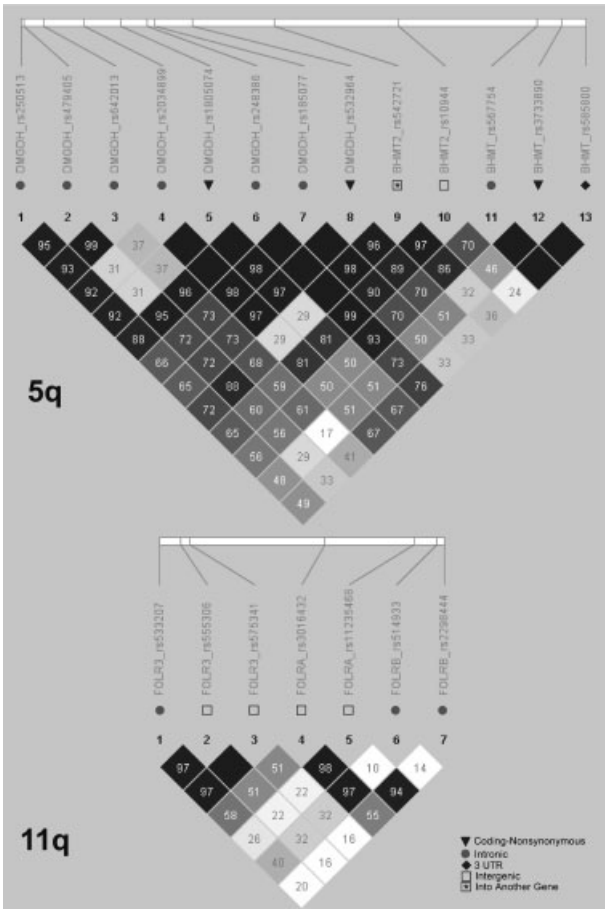

Fig. 2. 5q and 11q LD region Haploview LD plots. D' values are indicated in the pair wise squares. Shading is according to the alternative D'/LOD color scheme with darker shades indicating higher D' values if the LOD score is ≥ 2. Each SNP's functional status is indicated by the same symbols used in Figure 1. 168 × 253 mm (300 × 300 DPI)

little evidence of P-values in excess of what would be expected by chance (the dashed line). In the "CPO and Folate" panel, there is a cluster of genes carried by the child suggesting possible associations (in the high-folic acid stratum *FOLH1* $P = 0.0008$, *SHMT2* $P = 0.0089$, and *CTH* $P = 0.040$; in the low-folic acid stratum LD region Chr11 $P = 0.013$ and *SHMT1* $P = 0.015$). *MTHFR*, the most widely studied folate metabolizing gene, has a maternal genetic effect weakly associated with CPO in the high-folic acid stratum ($P = 0.044$).

Tables II and III provide individual SNP P-values and proposed risk haplotype alleles for the eight folate/one-carbon genes or LD regions and the four vitamin A genes with overall P-values less than 0.05. Complete results for all SNPs in every gene or each LD region are provided in Supplemental Tables III and IV.

Pathway-wide significance tests included all the polymorphisms in each pathway for all eight strata. In CPO there was marginal evidence of a maternal effect when mothers took $< 400\,\mu g$ of folic acid ($P = 0.0075$) and for a child effect in the $400^{+}\,\mu g$ mothers ($P = 0.0325$). In the

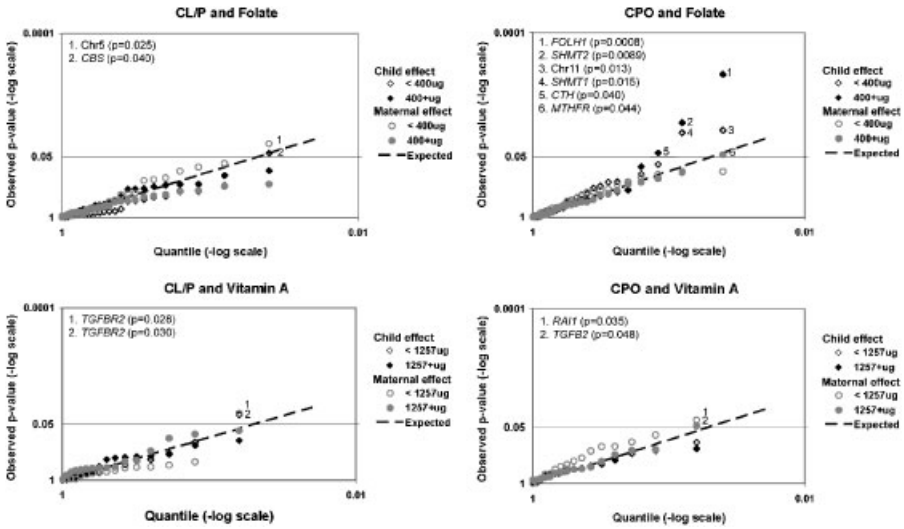

Fig. 3. Quantile-quantile plot of the observed *P*-values (-log scale) versus an expected uniform distribution under the null (for 20 independent tests we expect one test to have a *P*-value <0.05). CL/P and CPO are plotted separately for both folate-related genes and vitamin A-related genes. Each plot contains the TRIMM sum_logP results for each gene or LD region for child (diamond) or maternal (circle) genetic effects stratified by periconceptional vitamin supplementation $\pm 400\,\mu g$ of folic acid or $\pm 1{,}257\,\mu g$ of total vitamin A intake (low-vitamin, open symbols; high-vitamin, filled symbols). The expected null distribution is indicated by the dashed diagonal line (points above that line represent more significance than expected). For all results with observed *P*-values <0.05 (above the horizontal line), the gene or LD region and the associated *P*-value are detailed in Tables II and III. Complete results for each pathway, gene, LD region, and SNP are included in Supplemental Tables III–V. 147 × 239 mm (300 × 300 DPI)

vitamin A pathway, there was also a borderline maternal effect for CPO in the $<1{,}257\,\mu g$ group ($P = 0.0242$). The 13 other unadjusted pathway-wide *P*-values were >0.05 (Supplemental Table V).

DISCUSSION

Numerous studies have attempted to associate vitamin metabolism gene polymorphisms with oral facial clefts, often with conflicting results [Boyles et al., 2008; Chevrier et al., 2007; Jugessur et al., 2003a, b; Martinelli et al., 2006; Mitchell et al., 2003; Mostowska et al., 2006; Rubini et al., 2005; Scapoli et al., 2005; Shaw et al., 1998; van Rooij et al., 2003; Zhu et al., 2005]. Here, we found no evidence of a role for these gene variants in the risk of clefts, after accounting for the large number of tests performed. The strongest associations were between folate/one-carbon pathway genes and CPO, which is unexpected given the lack of epidemiologic evidence that folic acid supplementation prevents CPO in this population [Wilcox et al., 2007]. Similarly, there was no evidence of an association of genes related to metabolism of vitamin A with CL/P or CPO, either independently or in conjunction with lower vitamin A intake.

This article provides the first application of the TRIMM approach for genetic data analysis of a metabolic pathway. TRIMM is a versatile method for triad family data in that there is no limit to the number of SNPs that can be included, and no requirement of HWE. Prior specification of haplotypes or phase inference is also not necessary. TRIMM can evaluate maternal effects, which is especially important in birth defects where the maternal genes can contribute to the fetal in utero environment. Software for implementing TRIMM is available for the R computing environment (www.niehs.nih.gov/research/atniehs/labs/bb/staff/weinberg/index.cfm#downloads).

TRIMM is a powerful method for this type of data, but it does not estimate a meaningful risk parameter such as a relative risk. Moreover, a formal test of interaction cannot be performed. The nominated risk-haplotype-tagging alleles may not represent a true single haplotype because phase is not inferred from the data, but it may mark a jointly relevant set of marker alleles. It is theoretically possible that exactly complementary haplotypes that are both protective (or both risk-conferring) could cancel each other out, generating a null-value difference vector.

Considering the analysis more broadly, the unified approach to pathway-wide analysis presented here may be applicable to other large data sets with candidate genes and strong prior expectations about gene-environment interactions. A combined pathway-wide approach has the possibility of identifying synergistic relationships between genes that might be missed by single SNP analysis. Such studies would require evidence of an environmental risk factor associated with a group of biologically related genes and evidence of a multigenic model of disease inheritance.

We found multiple SNPs that contributed to a gene or LD region's overall significance of association. As shown in Table II several genes had multiple SNPs in the nominated risk-haplotype contributing to their overall *P*-value: *FOLH1* (3 SNPs), Chr5 (4 SNPs in *DMGDH*), and *CBS* (3 SNPs). In the case of *FOLH1*, the overall *P*-value was more significant than all of the individual SNP *P*-values. However, there were also cases where only one SNP was individually significant and the addition of other SNPs led to a less significant overall *P*-value: Chr11 (strongest SNP in *FOLR2*), *CTH*, and *MTHFR*.

TABLE II. Folate/one-carbon associated genes

Gene/LD region (Sum_logP)	Cleft Type	Effect	Folic acid ±400 μg	Gene (if in an LD region)	SNP	Type	max Z^2 *P*-value	Risk haplotype
FOLH1	CPO	Child	400+		rs6485963	near	1	
(0.0008)					rs11040270	ig	1	
					rs7113251	Int	1	
					rs202720	Int	0.011*	1
					rs10839236	Int	0.006*	1
					rs202676	NS	0.0064*	1
SHMT2 (0.0089)	CPO	Child	400+		rs7311958	near	0.0089*	1
Chr11q	CPO	Child	<400	*FOLR3*	rs533207	Int	0.581	
(0.013)				*FOLR3*	rs555306	ig	0.725	
				FOLR3	rs575341	ig	0.725	
				FOLR1	rs3016432	ig	0.309	
				FOLR1	rs11235468	ig	0.252	
				FOLR2	rs514933	Int	0.416	
				FOLR2	rs2298444	Int	0.003*	1
SHMT1	CPO	Child	<400		rs2168781	Int	0.015*	1
(0.015)					rs7207306	Int	0.17	
Chr5q	CL/P	Mom	<400	*DMGDH*	rs250513	Int	0.19	
(0.025)				*DMGDH*	rs479405	Int	0.01*	1
				DMGDH	rs642013	Int	0.127	
				DMGDH	rs2034899	Int	0.127	
				DMGDH	rs1805074	NS	0.0012*	1
				DMGDH	rs248386	Int	0.2675	
				DMGDH	rs185077	Int	0.1882	
				DMGDH	rs532964	NS	0.013*	1
				BHMT2	rs542721[a]	ig	0.033*	1
				BHMT2	rs10944	ig	0.139	
				BHMT1	rs567754	Int	0.29	
				BHMT1	rs3733890	NS	0.768	
				BHMT1	rs585800	UTR	0.13	
CBS	CL/P	Child	400+		rs4920037	Int	0.008*	1
(0.040)					844ins68[b]	ins	1	
					rs234705	Int	0.012*	1
					rs234706	Syn	0.121	
					rs234709	Int	0.081*	1
CTH	CPO	Child	400+		rs681475	Int	1	
(0.040)					rs1145920	Int	1	
					rs663649	Int	0.02*	1
					rs515064	Int	0.158	
					rs1021737	NS	0.119	
MTHFR	CPO	Mom	400+		rs4845877	ig	1	
(0.044)					rs1476413	Int	1	
					rs1801131	NS	0.02*	2
					rs1801133[c]	NS	0.637	
					rs3737964	ig	1	
					rs12404124	ig	0.362	

*Individual SNP max Z^2 *P*-value <0.05.
[a]Although selected for being in the region of *BHMT2*, this SNP actually lies in a *DMGDH* intron.
[b]Sixty-eight base pair insertion at position 844 in *CBS*.
[c]The SNP changes the protein to a thermolabile form (also referred to a C677T). It is the most widely studied SNP in folate metabolism.
Individual polymorphism max Z^2 *P*-values for all folate/one-carbon metabolizing genes or LD regions generating an overall sum_logP *P*-value <0.05. Risk haplotype alleles are coded 1 for major or 2 for minor allele. Functional type of polymorphism is indicated by the abbreviations: near, near the assigned gene, but actually in another gene; ig, intergenic; Int, intronic; NS, coding, nonsynonymous; UTR, 3′ untranslated region—there were no 5′ UTR SNPs; ins, insertion; and Syn, coding, synonymous.

The strongest association in this study was between several SNPs in *FOLH1* and risk of CPO in mothers who took at least 400 μg of folic acid. Even with the large number of tests and little evidence that folic acid prevents CPO, we cannot entirely dismiss the possibility that this observed association is real. Formerly called *GCP2*

TABLE III. Vitamin A associated genes

Gene (Sum_logP)	Cleft Type	Effect	Vitamin A ±1,257 μg	SNP	max Z^2 *P*-value	Risk haplotype
TGFBR2	CL/P	Mom	<1257	rs1835538	0.12	
(0.028)				rs4522809	0.0092*	2
				rs1155708	0.917	
				rs3773634	0.638	
				rs876688	0.927	
TGFBR2	CL/P	Child	<1257	rs1835538	0.69[a]	
(0.030[a])				rs4522809	0.0069*[a]	1
				rs1155708	1[a]	
				rs3773634	0.703[a]	
				rs876688	0.929[a]	
RAI1	CPO	Mom	<1257	rs11656775	0.031*	1
(0.035)				rs7224617	0.616	
TGFB2	CPO	Mom	1257^{+}	rs6658835	0.589	
(0.048)				rs2027566	1	
				rs2799090	0.019*	1
				rs2799103	0.142	
				rs2000220	0.545	
				rs2796813	0.443	

*Individual SNP max Z^2 *P*-value <0.05

[a]Families with missing genotypes were not used due to evidence of a maternal effect.

Individual SNP max Z^2 *P*-values for all vitamin A metabolizing genes generating an overall sum_logP *P*-value <0.05. Risk haplotype alleles are coded 1 for major or 2 for minor allele. All SNPs included are intronic.

(glutamate carboxypepidase II), *FOLH1* (folate hydrolase) encodes an intestinal brush border membrane protein that digests polyglutamylated folates into monoglutamyl folates [OMIM * 600934]. A polymorphism in *FOLH1* has been associated with low folate and high homocysteine levels possibly via decreased absorption of dietary folate from the intestines [Devlin et al., 2000], however, a larger study associated this polymorphism with high folate and low total homocysteine [Halsted et al., 2007]. There are no previously published genetic association studies of *FOLH1* and oral facial clefts, and so we are unable to compare our results with data from other similar studies.

We have provided detailed information on all our results, which may be useful for future meta-analyses of folate/one-carbon and vitamin A polymorphisms and facial clefts (Supplemental Tables III, IV). Even so, we suspect that further analyses are unlikely to yield strong results, given the lack of evidence so far among this wide array of vitamin metabolism-related genes. Ungenotyped variants, other-related genes, or epigenetic effects may also play a role, but perhaps the simplest explanation for these results is that the genetic contribution to oral facial clefts is independent of pathways by which vitamins provide protection from oral facial clefts.

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health Grants (DE085592, RO1 DE-11948-04, N01-HG-65403, P50 DE-16215, R37 DE-0559), the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (Z01 ES049027-11, Z01 ES040007); the Research Council of Norway (166026/V50); the Foundation to promote research into functional vitamin B12-deficiency; the Freia Foundation; the Throne-Holst Foundation; and the thematic area of perinatal nutrition, Faculty of Medicine, University of Oslo, Oslo, Norway. Genotyping services were provided by the Center for Inherited Disease Research (CIDR), which is fully funded through a federal contract from the National Institutes of Health to The Johns Hopkins University (N01-HG-65403). The authors would also like to acknowledge the valuable contributions of Andrew Lidral, Bridget Riley, Adela Mansilla, Kathy Frees, Corinne Boehm, Kim Doheny, and Ivy McMullen to the overall cleft candidate gene project.

REFERENCES

Abbott BD, Best DS, Narotsky MG. 2005. Teratogenic effects of retinoic acid are modulated in mice lacking expression of epidermal growth factor and transforming growth factor-alpha. Birth Defects Res A Clin Mol Teratol 73:204–217.

Badovinac RL, Werler MM, Williams PL, Kelsey KT, Hayes C. 2007. Folic acid-containing supplement consumption during pregnancy and risk for oral clefts: a meta-analysis. Birth Defects Res A Clin Mol Teratol 79:8–15.

Baroni T, Bellucci C, Lilli C, Pezzetti F, Carinci F, Becchetti E, Carinci P, Stabellini G, Calvitti M, Lumare E, Bodo M. 2006. Retinoic acid, GABA-ergic, and TGF-beta signaling systems are involved in human cleft palate fibroblast phenotype. Mol Med 12:237–245.

Barrett JC, Fry B, Maller J, Daly MJ. 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.

Bille C, Olsen J, Vach W, Knudsen VK, Olsen SF, Rasmussen K, Murray JC, Andersen AM, Christensen K. 2007. Oral clefts and life style factors—a case-cohort study based on prospective Danish data. Eur J Epidemiol 22:173–181.

Boyles AL, Wilcox AJ, Taylor JA, Meyer K, Fredriksen A, Ueland PM, Drevon CA, Vollset SE, Lie RT. 2008. Folate and one-carbon metabolism gene polymorphisms and their associations with oral facial clefts. Am J Med Genet A 146:440–449.

Brown NL, Knott L, Halligan E, Yarram SJ, Mansell JP, Sandy JR. 2003. Microarray analysis of murine palatogenesis: temporal expression of genes during normal palate development. Dev Growth Differ 45:153–165.

Cai J, Ash D, Kotch LE, Jabs EW, Attie-Bitach T, Auge J, Mattei G, Etchevers H, Vekemans M, Korshunova Y, Tidwell R, Messina DN, Winston JB, Lovett M. 2005. Gene expression in pharyngeal arch 1 during human embryonic development. Hum Mol Genet 14: 903–912.

Chevrier C, Perret C, Bahuau M, Zhu H, Nelva A, Herman C, Francannet C, Robert-Gnansia E, Finnell RH, Cordier S. 2007. Fetal and maternal MTHFR C677T genotype, maternal folate intake and the risk of nonsyndromic oral clefts. Am J Med Genet A 143:248–257.

Clementi M, Tenconi R, Forabosco P, Calzolari E, Milan M. 1997. Inheritance of cleft palate in Italy. Evidence for a major autosomal recessive locus. Hum Genet 100:204–209.

Devlin AM, Ling EH, Peerson JM, Fernando S, Clarke R, Smith AD, Halsted CH. 2000. Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia. Hum Mol Genet 9:2837–2844.

Finnell RH, Shaw GM, Lammer EJ, Brandl KL, Carmichael SL, Rosenquist TH. 2004. Gene-nutrient interactions: importance of folates and retinoids during early embryogenesis. Toxicol Appl Pharmacol 198:75–85.

Gong SG, Gong TW, Shum L. 2005. Identification of markers of the midface. J Dent Res 84:69–72.

Halsted CH, Wong DH, Peerson JM, Warden CH, Refsum H, Smith AD, Nygard OK, Ueland PM, Vollset SE, Tell GS. 2007. Relations of glutamate carboxypeptidase II (GCPII) polymorphisms to folate and homocysteine concentrations and to scores of cognition, anxiety, and depression in a homogeneous Norwegian population: the Hordaland Homocysteine Study. Am J Clin Nutr 86: 514–521.

Johansen AM, Lie RT, Wilcox AJ, Andersen LF, Drevon CA. 2008. Maternal dietary intake of vitamin A and risk of orofacial clefts: a population-based case-control study in Norway. Am J Epidemiol 167:1164–1170.

Jugessur A, Murray JC. 2005. Orofacial clefting: recent insights into a complex trait. Curr Opin Genet Dev 15:270–278.

Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, Vindenes HA, Abyholm F. 2003a. Variants of developmental genes (TGFA, TGFB3, and MSX1) and their associations with orofacial clefts: a case-parent triad analysis. Genet Epidemiol 24: 230–239.

Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, Vindenes HA, Abyholm FE. 2003b. Cleft palate, transforming growth factor alpha gene variants, and maternal exposures: assessing gene-environment interactions in case-parent triads. Genet Epidemiol 25:367–374.

Jugessur A, Wilcox AJ, Lie RT, Murray JC, Taylor JA, Ulvik A, Drevon CA, Vindenes HA, Abyholm FE. 2003c. Exploring the effects of methylenetetrahydrofolate reductase gene variants C677T and A1298C on the risk of orofacial clefts in 261 Norwegian case-parent triads. Am J Epidemiol 157: 1083–1091.

Krapels IP, van Rooij IA, Ocke MC, van Cleef BA, Kuijpers-Jagtman AM, Steegers-Theunissen RP. 2004. Maternal dietary B vitamin intake, other than folate, and the association with orofacial cleft in the offspring. Eur J Nutr 43:7–14.

Lidral AC, Moreno LM. 2005. Progress toward discerning the genetics of cleft lip. Curr Opin Pediatr 17:731–739.

Martin ER, Monks SA, Warren LL, Kaplan NL. 2000. A test for linkage and association in general pedigrees: the pedigree disequilibrium test. Am J Hum Genet 67:146–154.

Martinelli M, Scapoli L, Palmieri A, Pezzetti F, Baciliero U, Padula E, Carinci P, Morselli PG, Carinci F. 2006. Study of four genes belonging to the folate pathway: transcobalamin 2 is involved in the onset of non-syndromic cleft lip with or without cleft palate. Hum Mutat 27:294.

Meyer K, Fredriksen A, Ueland PM. 2004. High-level multiplex genotyping of polymorphisms involved in folate or homocysteine metabolism by matrix-assisted laser desorption/ionization mass spectrometry. Clin Chem 50:391–402.

Mitchell LE, Murray JC, O'Brien S, Christensen K. 2003. Retinoic acid receptor alpha gene variants, multivitamin use, and liver intake as risk factors for oral clefts: a population-based case-control study in Denmark, 1991–1994. Am J Epidemiol 158:69–76.

Mostowska A, Hozyasz KK, Jagodzinski PP. 2006. Maternal MTR genotype contributes to the risk of non-syndromic cleft lip and palate in the Polish population. Clin Genet 69:512–517.

Mukhopadhyay P, Greene RM, Zacharias W, Weinrich MC, Singh S, Young Jr WW, Pisano MM. 2004. Developmental gene expression profiling of mammalian, fetal orofacial tissue. Birth Defects Res A Clin Mol Teratol 70:912–926.

Murray JC, Daack-Hirsch S, Buetow KH, Munger R, Espina L, Paglinawan N, Villanueva E, Rary J, Magee K, Magee W. 1997. Clinical and epidemiologic studies of cleft lip and palate in the Philippines. Cleft Palate Craniofac J 34:7–10.

Nguyen RH, Wilcox AJ, Moen BE, McConnaughey DR, Lie RT. 2007. Parent's occupation and isolated orofacial clefts in Norway: a population-based case-control study. Ann Epidemiol 17: 763–771.

Palomino H, Cerda-Flores RM, Blanco R, Palomino HM, Barton SA, de Andrade M, Chakraborty R. 1997. Complex segregation analysis of facial clefting in Chile. J Craniofac Genet Dev Biol 17:57–64.

Rubini M, Brusati R, Garattini G, Magnani C, Liviero F, Bianchi F, Tarantino E, Massei A, Pollastri S, Carturan S, Amadori A, Bertagnin E, Cavallaro A, Fabiano A, Franchella A, Calzolari E. 2005. Cystathionine beta-synthase c.844ins68 gene variant and non-syndromic cleft lip and palate. Am J Med Genet A 136: 368–372.

Scapoli C, Collins A, Martinelli M, Pezzetti F, Scapoli L, Tognon M. 1999. Combined segregation and linkage analysis of non-syndromic orofacial cleft in two candidate regions. Ann Hum Genet 63:17–25.

Scapoli L, Marchesini J, Martinelli M, Pezzetti F, Carinci F, Palmieri A, Rullo R, Gombos F, Tognon M, Carinci P. 2005. Study of folate receptor genes in nonsyndromic familial and sporadic cleft lip with or without cleft palate cases. Am J Med Genet A 132: 302–304.

Sebastiani P, Lazarus R, Weiss ST, Kunkel LM, Kohane IS, Ramoni MF. 2003. Minimal haplotype tagging. Proc Natl Acad Sci USA 100:9900–9905.

Shaw GM, Wasserman CR, Murray JC, Lammer EJ. 1998. Infant TGF-alpha genotype, orofacial clefts, and maternal periconceptional multivitamin use. Cleft Palate Craniofac J 35:366–370.

Shi M, Umbach DM, Weinberg CR. 2007. Identification of risk-related haplotypes with the use of multiple SNPs from nuclear families. Am J Hum Genet 81:53–66.

Sivertsen A, Wilcox AJ, Skjaerven R, Vindenes HA, Abyholm F, Harville E, Lie RT. 2008. Familial risk of oral clefts by morphological type and severity: population based cohort study of first degree relatives. Br Med J 336:432–434.

Soprano DR, Soprano KJ. 1995. Retinoids as teratogens. Annu Rev Nutr 15:111–132.

van Rooij IA, Vermeij-Keers C, Kluijtmans LA, Ocke MC, Zielhuis GA, Goorhuis-Brouwer SM, van der Biezen JJ, Kuijpers-Jagtman AM, Steegers-Theunissen RP. 2003. Does the interaction between maternal folate intake and the methylenetetrahydrofolate reductase polymorphisms affect the risk of cleft lip with or without cleft palate? Am J Epidemiol 157:583–591.

van Rooij IA, Ocke MC, Straatman H, Zielhuis GA, Merkus HM, Steegers-Theunissen RP. 2004. Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. Prev Med 39:689–694.

Vieira AR, Romitti PA, Orioli IM, Castilla EE. 2003. Inheritance of cleft palate in South America: evidence for a major locus recessive. Orthod Craniofac Res 6:83–87.

Wigginton JE, Cutler DJ, Abecasis GR. 2005. A note on exact tests of Hardy–Weinberg equilibrium. Am J Hum Genet 76:887–893.

Wilcox AJ, Lie RT, Solvoll K, Taylor J, McConnaughey DR, Abyholm F, Vindenes H, Vollset SE, Drevon CA. 2007. Folic acid supplements and risk of facial clefts: national population based case-control study. Br Med J 334:464–470.

Zhu H, Curry S, Wen S, Wicker NJ, Shaw GM, Lammer EJ, Yang W, Jafarov T, Finnell RH. 2005. Are the betaine-homocysteine methyltransferase (BHMT and BHMT2) genes risk factors for spina bifida and orofacial clefts? Am J Med Genet A 135: 274–277.