Genetic Variation in the One-Carbon Transfer Pathway and Ovarian Cancer Risk

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Abstract

Dysfunction in enzymes involved in one-carbon (1-C) metabolism can lead to increased chromosomal strand breaking and abnormal methylation patterns, which are both associated with cancer risk. Availability of 1-C units may modify risk. We investigated the association of single-nucleotide polymorphisms (SNP) in 21 genes in the 1-C transfer pathway among 829 Caucasian cases with primary epithelial ovarian cancer and 941 frequency-matched unaffected controls enrolled at Mayo Clinic (Rochester, MN) and Duke University (Durham, NC) and examined risk modification by multivitamin supplement use. Multivariable-adjusted SNP-specific logistic regression and haplotype analyses were done for 180 SNPs and false positive report probabilities (FPRP) were calculated. Each copy of the minor allele in *SHMT1* intron 5 A>G (rs9909104) was associated with epithelial ovarian cancer [odds ratio (OR), 1.2; 95% confidence interval (95% CI), 1.0–1.4; *P* trend = 0.02; FPRP = 0.16] and a 5-SNP *SHMT1* haplotype was associated with decreased risk (*P* = 0.01; FPRP = 0.09). Three SNPs in *DNMT3A* were associated with risk among multivitamin supplement users: 3′ untranslated region (UTR) C>G (rs13420827: OR, 0.8; 95% CI, 0.6–1.0; *P* interaction = 0.006; FPRP = 0.54), intron 6 G>A (rs11887120: OR, 0.8; 95% CI, 0.7–1.0; *P* interaction = 0.007; FPRP = 0.57), and intron 22 A>T (rs11695471: OR, 1.2; 95% CI, 1.0–1.5; *P* interaction = 0.01; FPRP = 0.66). These data extend previous findings from other cancers of a role for *SHMT1* in ovarian cancer, and provide evidence that SNPs in methylation and DNA synthesis reactions are associated with risk of ovarian cancer. Interventions with modifiable factors such as multivitamin intake may reduce risk. [Cancer Res 2008;68(7):2498–506]

Introduction

Ovarian cancer is the eighth most common cancer among U.S. women, with 22,430 newly diagnosed cases and 15,280 deaths estimated in 2007 (1). The few known risk factors are either reproductive related [decreased risk from oral contraceptive use (2), parity (3), and long-term breastfeeding (3)] or represent inherited mutations in a few high-risk, high-penetrance genes (e.g., *BRCA1*; ref. 4). Empirical calculations suggest (5) that a modest number (≤20) of common predisposing genetic variants (each with prevalence ≥25%) could explain 50% of the burden of a disease in the population, even if the individual genotype associations are relatively small (e.g., relative risk, 1.2–1.5). These common variants could plausibly interact with common environmental exposures to alter risk among a substantial proportion of individuals.

Perturbation in one-carbon (1-C) metabolism can have pleiotropic consequences that may lead to tumor initiation and progression. One-carbon transfer reactions are important for DNA synthesis, particularly for rapidly dividing cells (6), and also for the biosynthesis of *S*-adenosyl methionine, an essential supplier of methyl groups for many compounds including DNA (6). Because folate is a basic component of cell metabolism and is integral to the 1-C transfer pathway, it is not surprising that folate or methyl-donor nutrient deficiency can lead to gene-specific (7) or global (8) DNA hypomethylation, increased chromosomal strand breaking (9), and alone can act as complete carcinogens or as effective tumor promoters after chemical initiation (10, 11).

Common single-nucleotide polymorphisms (SNP) in genes encoding 1-C transfer-associated enzymes that rely on folate or methyl-donor nutrients may imitate the outcome of vitamin deficiency by providing insufficient 1-C moieties for methylation or DNA synthesis (12–15). Perhaps the most studied is *MTHFR*: two copies of the rare allele are associated with modest decreased risks of colon cancer, which is most evident among those with higher folate intake (16). Polymorphisms in genes in 1-C metabolism have not been examined extensively with ovarian cancer, but can complement and strengthen findings from the dietary-only association studies, which are inconsistent (17, 18); identify novel variants worthy of additional interrogation; locate associated region(s) for future fine-mapping; and lead to functional and interventional studies that examine risk modification within the context of exposure to high or low intakes of folate or methyl-donor nutrients.

Here, we report findings from the association of 180 tag SNPs and putative functional common SNPs in genes in the 1-C transfer and methylation-related pathways with risk of ovarian cancer using data from two ongoing case-control studies. We also examined effect modification by multivitamin supplement use as an estimate of B-vitamin intake.

Materials and Methods

Study Design and Population

Subjects participated in two ongoing case-control studies of epithelial ovarian cancer initiated in January 2000 at Mayo Clinic (Rochester, MN) and in May 1999 at Duke University (Durham, NC). Written informed consent was obtained from all participants. For the current analyses, we included participants enrolled during the period June 1999 to March 2006. The

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Institutional Review Board at both sites approved the study protocols. Details of the study design are described elsewhere (19) and briefly outlined below.

Mayo Clinic sample. Clinic attendance formed the sampling frame for Mayo cases and controls. Mayo cases were women ages >20 y with histologically confirmed incident epithelial ovarian cancer (borderline or invasive) and enrolled in the study within 1 y of date of diagnosis. Cases lived in the six-state region that defines the primary service population of Mayo Clinic (Minnesota, Iowa, Wisconsin, Illinois, North Dakota, and South Dakota) and composes ~85% of all ovarian cancer cases seen at Mayo Clinic. Controls without ovarian cancer and who had at least one ovary intact were frequency matched on race, age (5-y age categories), and region of residence to cases. Controls were recruited from the outpatient practice of the Divisions of General Internal Medicine and Primary Care Internal Medicine at Mayo Clinic. Women were seen for medical evaluations for many conditions including those typical of older Americans such as hypertension, diabetes, hyperlipidemia, and coronary artery disease. The response was 83% among cases and 74% among controls.

Duke University sample. A 48-county area of North Carolina formed the sampling frame for Duke cases and controls. Duke cases were women between the ages of 20 and 74 y, with histologically confirmed primary epithelial ovarian cancer (borderline or invasive). Cases were identified using the North Carolina Central Cancer Registry rapid case ascertainment system. Controls without ovarian cancer and who had at least one ovary intact were identified from the same 48-county region as the cases using list-assisted random digit dialing. Controls were frequency matched to cases on race and age (5-y age categories). The response was 75% among eligible cases and 64% among the controls.

Risk Factor Questionnaire

Information on demographic data and known and suspected ovarian cancer risk factors was collected through in-person interviews at both sites using similar questionnaires. In January 2003, the Mayo questionnaire was expanded with questions about "regular multivitamin" intake defined as ≥4 pills/wk during the previous year for controls and 1 y before cancer diagnosis for the cases. The Duke questionnaire elicited this information from study start with three possible responses to ≥1 pill/wk ("yes, regularly," "yes irregularly," or "no"). We defined users as those who responded "yes regularly" during the past 5 y for controls and in the 5 y before diagnosis for cases. A common data dictionary was developed for covariates to allow combined analysis of data from both sites.

Biospecimen Collection and Processing

DNA was extracted from blood using the Gentra AutoPure LS Purgene salting out methods (Gentra). Due to limited quantity of available DNA from Duke subjects, we performed whole genome amplification (WGA) on all Duke samples (n = 1,282) as a means to enrich DNA quantities. WGA DNA was prepared from 200-ng genomic DNA using the REPLI-G WGA protocol (Qiagen, Inc.). Quantities of 250-ng genomic and WGA DNA were adjusted to 50 ng/μL before genotyping and verified using PicoGreen dsDNA quantitation kit (Molecular Probes, Inc.). The samples were bar coded to ensure accurate and reliable sample processing and storage.

Gene, SNP, and TagSNP Selection

Genes encoding proteins in the 1-C transfer and methylation-related pathways were identified from literature searches and public databases (e.g., Kegg). We focused on genes that have known or suggestive data of associations with other diseases including cancer (12, 13, 20–23), deficiency syndromes (24, 25), embryonic development (26), neural tube defects (27), cardiovascular disease (28), or functional studies (29), or whose gene product participated in a rate-limiting step, irreversible direction, affected ligand binding or generated important intermediate substrates to that pathway, with the expectation that polymorphisms in these genes would have the potential to impart the greatest functional effect on outcome according to current knowledge. Twenty-one genes (*AHCYL1, ALDH1L1, DHFR, DNMT1, DNMT3A, DNMT3B, DPYD, FOLR1, MAT2B, MBD4, MGMT, MTHFD1, MTHFD1L, MTHFD2, MTHFR, MTHFS, MTR, MTRR, SHMT1, SLC19A1*, and *TYMS*) were selected for their role in 1-C transfer and metabolism, including participation in the folate and methionine cycles, methylation, purine and pyrimidine synthesis, and folate transport.

All SNPs within the 21 candidate genes 5 kb of the largest cDNA isoform (genome build 35) were selected from unrelated Caucasian samples within the HapMap Consortium release 21[6] (30), Perlegen Sciences[7] (31), SeattleSNPs,[8] and Panel 2 of the National Institute for Environmental Health Science SNPs.[9] We applied the ldSelect program (32) to bin SNPs with minor allele frequency ≥0.05 and pairwise linkage disequilibrium (LD) threshold of r^2 ≥0.80. Following binning, we selected tagSNPs for analysis from the source with the greatest number of SNPs with minor allele frequency ≥0.05 and the greatest number of LD bins that also met criteria for predicted likelihood of successful genotyping using the Illumina Golden Gate Assay quality score metrics. We also included all putative functional SNPs (within 1 kb upstream, 5′ UTR, 3′ UTR, or nonsynonymous) with minor allele frequency ≥0.05 identified in Ensembl release 34. Nucleotide positions for SNPs were calculated as the difference between the gene start coordinate and SNP coordinate using Ensembl release 47. The 21 1-C transfer and methylation-related genes contributed 153 tagSNPs (98% from HapMap) representing 2,710 individual SNPs and 35 additional putative functional SNPs for a total of 188 SNPs. Henceforth, we collectively refer to both tag SNPs and functional SNPs as "SNPs" unless otherwise clarified.

Genotyping

Mayo and Duke samples were plated separately in the Mayo Clinic Cancer Center Genotyping Shared Resource, with cases and controls randomly mixed within each plate. For the Mayo genomic DNA, each plate contained two subject DNAs in duplicate, a CEPH trio, and three known laboratory quality control samples. For the Duke WGA DNA, 88 samples were duplicated with an aliquot of the same WGA preparation, whereas 15 were duplicated with a separate WGA preparation. In addition, 124 individuals with WGA samples had sufficient genomic DNA for genotyping to understand the performance of WGA compared with genomic DNA (33).

Genotyping of 1,086 genomic and 1,282 WGA DNA samples (total: 2,368 including duplicates and laboratory controls) was done at Mayo Clinic using the Illumina GoldenGate BeadArray assay and BeadStudio software for automated genotype clustering and calling according to a standard protocol (34).

Quality Control and Exclusions

Samples with Illumina GenCall scores (a metric of reliability of called genotypes generated by the BeadStudio software) <0.25 or call rates <90%, and SNPs with GenCall scores <0.4 or call rates <90%, failed immediately for both genomic and WGA DNA. Of 2,051 samples genotyped, 10 were found to be ineligible and were excluded and 74 samples failed. These consisted of 72 that clustered especially poorly and were therefore failed for every SNP and 2 confirmed sample errors, resulting in a final sample size of 1,967 subjects. Of 188 SNPs, 8 failed, leaving 180 SNPs available for analysis listed in Supplementary Table S1.

Among SNPs with an overall call rate ≥95%, concordance was 99.99% between duplicates of genomic DNA, 99.97% between duplicates of WGA DNA, and 99.16% between genomic and WGA DNA, indicating successful genotyping of WGA DNA for use in this study (33).

Statistical Analyses

The present analyses excluded 197 non-Caucasian subjects. Participants' genotypes were used to estimate allele frequencies. Among control subjects, genotypes were compared with those expected under Hardy-Weinberg equilibrium (HWE).

We compared the distribution of potential risk factors among cases and controls across study sites using ANOVA and χ^2 tests. Risk models were

[6] http://www.hapmap.org
[7] http://genome.perlegen.com
[8] http://pga.mbt.washington.edu
[9] http://www.egp.gs.washington.edu

adjusted for variables associated with ovarian cancer case-control status (see Table 2 footnotes), but no appreciable differences in risk estimates were observed without their inclusion. Pairwise LD between SNPs was estimated with r^2 values (35) using Haploview (36). Individual SNP associations for ovarian cancer risk were assessed using unconditional logistic regression to estimate odds ratios (OR) and 95% confidence intervals (95% CI). Association testing assumed an ordinal (log-additive) genotypic relationship with simple tests for trend, as well as separate comparisons of women with one copy and two copies of the minor allele to women with no copies (reference) using a 2-degree-of-freedom (*df*) test. Haplotype frequencies for each gene were estimated using all SNPs within the gene, and a global haplotype score test of no association between haplotypes and ovarian

Table 1. Characteristics of 1,770 Caucasian subjects, Mayo Clinic and Duke University, 1999–2006

Characteristic*	Mayo			Duke		
	Cases	Controls	$P^{\dagger}$	Cases	Controls	$P^{\dagger}$
n	385	462		444	479	
Age, y [mean (SD)]	59.9 (13.4)	60.0 (13.0)	0.91	54.6 (11.3)	54.8 (12.0)	0.82
Body mass index, kg/m^2						
<23	76 (20.5)	107 (24.8)	0.03	120 (27.8)	129 (27.6)	0.54
23–26	88 (23.2)	121 (28.0)		106 (24.5)	117 (25.1)	
26–29	96 (25.9)	110 (25.5)		88 (20.4)	110 (23.6)	
≥29	112 (30.3)	94 (21.8)		118 (27.3)	111 (23.8)	
Age at menarche, y						
<12	52 (18.2)	67 (15.8)	0.47	110 (24.8)	85 (17.7)	0.05
12	75 (26.3)	97 (22.9)		129 (29.1)	143 (29.9)	
13	78 (27.4)	124 (29.2)		106 (23.9)	139 (29.0)	
≥14	80 (28.1)	136 (32.1)		98 (22.1)	112 (23.4)	
Oral contraceptive use, mo						
Never	171 (47.6)	164 (38.6)	<0.001	137 (31.4)	147 (30.9)	0.65
1–48	94 (26.2)	90 (21.2)		133 (30.5)	134 (28.2)	
≥48	94 (26.2)	171 (40.2)		166 (38.1)	194 (40.8)	
Postmenopausal status						
Yes	262 (71.2)	327 (75.2)	0.20	302 (73.5)	316 (67.2)	0.04
Postmenopausal hormone use, mo						
Never	231 (63.1)	243 (58.4)	0.40	151 (35.0)	278 (59.5)	<0.001
1–60	63 (17.2)	79 (19.0)		168 (38.9)	99 (21.2)	
≥60	72 (19.7)	94 (22.6)		113 (26.2)	90 (19.3)	
Parity, *n*/age at first birth, y						
Nulliparous	68 (18.2)	64 (14.8)	0.07	95 (21.4)	62 (12.9)	0.01
1–2/≤20	28 (7.5)	25 (5.8)		56 (12.6)	56 (11.7)	
1–2/>20	102 (27.3)	128 (29.6)		171 (38.5)	212 (44.3)	
≥3/≤20	71 (19.0)	63 (14.5)		59 (13.3)	62 (12.9)	
≥3/>20	104 (27.9)	153 (35.3)		63 (14.2)	87 (18.2)	
Family history of ovarian cancer[‡]						
Yes	50 (13.4)	32 (7.3)	0.004	33 (7.4)	20 (4.2)	0.03
Smoking, pack-years						
None	227 (65.2)	279 (68.0)	0.42	245 (57.4)	248 (54.0)	0.60
≤20	69 (19.8)	83 (20.2)		102 (23.9)	120 (26.1)	
>20	52 (14.9)	48 (11.7)		80 (18.7)	91 (19.8)	
Education						
No diploma	23 (6.5)	19 (4.4)	<0.001	35 (7.9)	43 (9.0)	0.43
High school diploma	133 (37.7)	114 (26.1)		139 (31.3)	132 (27.6)	
Post-high school education	197 (55.8)	303 (69.5)		270 (60.8)	304 (63.5)	
Multivitamin use[§]						
Yes	105 (51.7)	271 (64.7)	0.002	231 (52.3)	238 (49.8)	0.45
Tumor histology, cases						
Serous	230 (59.7)			270 (60.8)		
Mucinous	28 (7.3)			52 (11.7)		
Endometrioid	64 (16.6)			56 (12.6)		
Clear cell	22 (5.7)			29 (6.5)		
Other	40 (10.4)			36 (8.1)		

* Data are counts (percentage) unless otherwise indicated. Counts do not total to 1,770 subjects due to missing data for some variables.

† Statistics are *t* test (continuous variables) and χ^2 test (categorical variables).

‡ In first- or second-degree relative.

§ At least 4 pills/wk during the previous year (Mayo subjects) or ≥1 pill/wk during the past 5 y (Duke subjects).

Table 2. Multivariable-adjusted ORs and 95% CIs between selected polymorphisms in genes in the 1-C metabolism pathway and ovarian cancer risk among 1,770 Caucasian subjects, Mayo Clinic and Duke University, 1999–2006

Gene/SNP rsID	Homozygotes common allele (Referent)		Heterozygotes			Homozygotes rare allele			Ordinal (Per rare allele)		
	Cases	Controls	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)	2*df P*	OR (95% CI)	*P* trend
AHCYL1											
17668350	684	806	141	127	1.4 (1.0–1.8)	3	8	0.4 (0.1–1.7)	0.04	1.2 (0.9–1.6)	0.11
DNMT3A											
13420827	552	602	234	308	0.8 (0.7–1.0)	38	28	1.5 (0.9–2.6)	0.03	1.0 (0.8–1.1)	0.68
DPYD											
1801265	463	585	321	318	1.3 (1.0–1.6)	44	38	1.4 (0.9–2.2)	0.04	1.2 (1.0–1.5)	0.01
MTHFD1											
1950902	552	586	250	306	0.9 (0.7–1.1)	17	37	0.5 (0.3–0.9)	0.06	0.8 (0.7–1.0)	0.04
2236225	229	288	421	481	1.1 (0.9–1.4)	174	166	1.3 (1.0–1.8)	0.15	1.1 (1.0–1.3)	0.05
11849530	508	512	273	375	0.7 (0.6–0.9)	48	53	0.9 (0.6–1.3)	0.02	0.8 (0.7–1.0)	0.03
MTHFS											
17284990	502	557	262	338	0.9 (0.7–1.1)	64	45	1.6 (1.1–2.5)	0.01	1.1 (0.9–1.2)	0.49
SHMT1											
9909104	437	539	317	340	1.2 (0.9–1.4)	73	61	1.5 (1.0–2.2)	0.09	1.2 (1.0–1.4)	0.02
SLC19A1											
3788205	422	434	331	409	0.8 (0.7–1.0)	75	98	0.8 (0.5–1.1)	0.10	0.9 (0.7–1.0)	0.04
TYMS											
495139	282	352	390	449	1.1 (0.8–1.3)	154	140	1.4 (1.0–1.8)	0.10	1.1 (1.0–1.3)	0.05

NOTE: Data were adjusted for age (<40, 40–49, 50–59, 60–69, 70+ y), state (Minnesota, Iowa, Wisconsin, Illinois, North Dakota/South Dakota, North Carolina), body mass index (<23, 23–25.9, 26–28.9, 29+ kg/m^2), postmenopausal hormone use (never, 1–60, 60+ mo), oral contraceptive use (never, 1–48, 48+ mo), and parity/age at first birth (nulliparous, 1–2/≤20, 1–2/>20, 3+/≤20, 3+/>20 y).

cancer risk was evaluated at the "gene level" by the method proposed by Schaid et al. (37). Individual haplotype associations compared each haplotype to all other haplotypes combined.

We also simultaneously modeled the comparison between controls and risk for each of the four main histologic subtypes of epithelial ovarian cancer (serous, endometrioid, clear cell, and mucinous) under the ordinal genetic model using polytomous logistic regression and tested for statistical heterogeneity of the SNP-ovarian cancer histology associations (38).

Interactions between multivitamin intake and genotype (and haplotypes for *SHMT1* and *MTR*) were evaluated for all SNPs under an ordinal genotypic relationship, where the association of a "fixed" genotype with ovarian cancer was assumed to depend on the "modifiable" exposure to multivitamin supplement use.

As an adjunct approach to identify genes (and therefore SNPs) that were significantly associated with ovarian cancer, we used principal components analysis to create orthogonal (e.g., uncorrelated) linear combinations of SNP minor allele counts that accounted for at least 90% of the variability in a gene. These were included in multivariable logistic regression models and tested for significance using a likelihood ratio test. By applying this method, we assumed that there would be residual correlation among SNPs (e.g., r^2 <0.8) that, when accounted for, would decrease the dimensionality of the data by reducing the number of independent degrees of freedom that composed the statistical test. Significant associations with ovarian cancer at both the individual SNP level and at the gene level using principal components were interpreted as supportive evidence for the individual SNP-ovarian cancer association.

To account for chance associations from multiple tests, we calculated the false positive report probability (FPRP; ref. 39), which depends on the prior probability that the SNP is associated with ovarian cancer, the power of the present study, and the observed *P* value. We set a FPRP threshold of <0.7 (e.g., ≤70% probability that the study hypotheses were falsely positive) as "noteworthy" for an initial study of a relatively rare tumor. Assuming a study power of 80%, we assigned a prior probability of 0.01 to detect an OR of 1.5 or 0.67 for a SNP that was significant at both the individual level and at the gene level or for a haplotype where the gene had a significant global haplotype score test, and to detect smaller ORs of 1.3 or 0.76 for SNP-multivitamin interactions with the expectation that there will be greater power to detect the gene effect among a homogeneous subset of the population exposed to multivitamin use (39). In addition, if an association with cancer was previously reported for a specific SNP, we calculated the FPRP using a higher prior probability of 0.1. We did this for *SHMT1* (40, 41).

Analyses were implemented using Haplo.stats,[10] SAS (SAS Institute, version 8, 1999), and S-Plus (Insightful Corp., version 7.05, 2005) software systems.

Results

All SNPs, their chromosomal locations, minor allele frequencies, and HWE statistics are listed in Supplementary Table S1. Fifteen SNPs showed departures from HWE among control subjects (P < 0.05); nine would be expected by chance. Although some investigators have discarded SNPs with statistical significance for HWE at P < 0.001 (42), we retained three SNPs in *MTR* at this level of significance. The minor allele frequencies among controls ranged from 0.02 to 0.49 and were similar across study sites. Cases (n = 829) and controls (n = 941) at both sites were somewhat different in the distribution of covariates (Table 1). A greater proportion of Mayo compared with Duke subjects had a family history of ovarian cancer and reported taking multivitamins.

[10] http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm

Despite these differences, cases were comparable across sites in distribution of tumor histology.

Multivariable-adjusted associations for 10 SNPs in eight genes that showed significance at $P \leq 0.05$ (ordinal or general model) are shown in Table 2. Of these, only SNPs in *DPYD* ($P = 0.05$) and *SHMT1* ($P = 0.03$) were significant at the gene level using principal components analysis (data not shown). Two copies of the minor allele in both *DPYD* Arg29Cys (rs1801265) and *SHMT1* intron 5 A>G (rs9909104) were associated with increased risk in a dose-response manner. The remaining SNPs (Table 2) also showed associations with ovarian cancer risk, but in the absence of a significant gene-level test. SNPs with nonsignificant associations are found in Supplementary Table S2.

Only the *SHMT1* and *MTR* genes were significant using global haplotype score tests for association with ovarian cancer risk (Table 3). Of five individual haplotypes estimated in *SHMT1*, the 5-SNP haplotype #1 accounting for 33% of all estimated haplotypes was associated with decreased risk ($P = 0.01$), whereas the 5-SNP haplotype #5 with 25% frequency was associated with increased risk ($P = 0.03$). Of 11 individual haplotypes estimated in *MTR*, the 8-SNP haplotype #1 with 12% frequency was associated with decreased risk ($P = 0.02$), whereas the 8-SNP haplotype #11 with 2% frequency was associated with increased risk ($P = 0.01$). Five *MTR* loci [intron 4 A>G (rs12759827), intron 5 C>T (rs4659724), 3′ UTR C>A (rs2853523), 3′ UTR C>T (rs1050993), and 3′ UTR G>T (rs6676866)] that composed the 8-SNP haplotypes had genotype distributions among control subjects that were significantly different than expected under HWE ($P < 0.002$; Supplementary Table S1). When Mayo and Duke samples were examined separately, all but one (intron 5 C>T [rs4659724], $P = 0.01$) of the eight SNPs were in HWE among Duke subjects; however, all SNPs remained out of HWE ($P < 0.001$) among Mayo subjects. Because haplotype inference assumes HWE, a spurious haplotype association is possible.

Analyses by histologic subtype revealed statistical heterogeneity ($P = 0.01$) for the association of *DNMT3B* intron 1 G>A (rs6119954) with ovarian cancer. Compared with controls, the ordinal genetic model estimated increased risk for endometrioid (OR, 1.6; 95% CI, 1.1–2.2; 120 cases) and clear cell (OR, 1.6; 95% CI, 1.0–2.8; 51 cases) tumors, but not for serous (OR, 0.9; 95% CI, 0.7–1.2; 500 cases) or mucinous (OR, 0.9; 95% CI, 0.5–1.4; 80 cases) tumors, although the findings may be from chance due to small numbers of cases.

SNP-specific associations with ovarian cancer and modified by multivitamin use under the ordinal genetic model are shown in Table 4. Among women who took multivitamins regularly, the per-minor allele risk was decreased for SNPs in *DNMT3A* [3′ UTR C>G

Table 3. Multivariable-adjusted haplotype analysis of genes in the 1-C metabolism pathway and ovarian cancer risk among 1,770 Caucasian subjects, Mayo Clinic and Duke University, 1999–2006

Haplotype		Global *P**	Estimated haplotype frequency	Score test †	Haplotype *P* ‡
No.	Allele combinations				
SHMT1§					
1	GTCAG	0.05	0.33	−2.56	0.01
2	ATCGG		0.01	−0.44	0.66
3	ACTAG		0.31	−0.01	0.99
4	GTCAA		0.10	1.21	0.23
5	GTCGG		0.25	2.14	0.03
MTR‖					
1	AGCTAATT	0.04	0.12	−2.29	0.02
2	AACCGCCG		0.14	−1.45	0.14
3	AGTCACCG		0.002	−0.69	0.49
4	AGCTACCG		0.004	−0.07	0.94
5	GGCTAATT		0.26	0.26	0.79
6	AGCCGCCG		0.04	0.40	0.69
7	AGTTACCG		0.38	0.79	0.43
8	AGTTAATT		0.01	1.03	0.30
9	AGTTACCT		0.002	1.07	0.28
10	GGCTACCG		0.01	1.17	0.24
11	AGCTACCT		0.02	2.47	0.01

NOTE: Data were adjusted for age (<40, 40–49, 50–59, 60–69, 70+ y), state (Minnesota, Iowa, Wisconsin, Illinois, North Dakota/South Dakota, North Carolina), body mass index (<23, 23–25.9, 26–28.9, 29+ kg/m^2), postmenopausal hormone use (never, 1–60, 60+ mo), oral contraceptive use (never, 1–48, 48+ mo), and parity/age at first birth (nulliparous, 1–2/≤20, 1–2/>20, 3+/≤20, 3+/>20 y).

* *P* value from the global score test of Schaid et al. (37) across haplotypes.

† Score statistics comparing haplotype of interest with all other haplotypes combined. Negative values imply decreased risk of ovarian cancer, whereas positive values imply increased risk.

‡ *P* value comparing haplotype of interest with all other haplotypes combined.

§ Haplotype-forming SNPs in *SHMT1* are rs921986 (G>A), rs12952556 (T>C), rs1979277 (C>T), rs9909104 (A>G), and rs2273026 (G>A). Different alleles between significant haplotypes are underlined.

‖ Haplotype-forming SNPs in *MTR* are rs12759827 (A>G), rs4659723 (G>A), rs4659724 (C>T), rs10925250 (T>C), rs1805087 (A>G), rs2853523 (C>A), rs1050993 (C>T), and rs6676866 (G>T). Different alleles between significant haplotypes are underlined.

(rs13420827) and intron 6 G>A (rs11887120)], *DNMT1* intron 23 C>T (rs9305012), and *MTHFR* 3′ UTR A>G (rs2184226), but risk was increased for SNPs in *DNMT3A* intron 22 A>T (rs11695471) and *MTHFD1* intron 17 C>T (rs17101854). Only the *DNMT3A* 3′ UTR C>G (rs13420827) was significantly associated with risk in main effects models (Table 2). Interaction associations for the remaining SNPs are found in Supplementary Table S3. In subsequent analyses that stratified *SHMT1* and *MTR* haplotypes by multivitamin use, the global haplotype score test was not significant among users (P = 0.06) or nonusers (P = 0.53) for *SHMT1*. For *MTR*, the global haplotype score test was significant among users (P = 0.03) but not among nonusers (P = 0.34). The individual 8-SNP *MTR* haplotype #1 (AGCTAATT) was associated with decreased risk among supplement users (P = 0.004; Supplementary Table S4).

The calculated FPRPs were below our preset value of 0.7 for the main effect of *SHMT1* intron 5 A>G (rs9909104; FPRP = 0.16) and for the 5-SNP *SHMT1* haplotype #1 (0.09 for GTCAG). FPRPs were also lower for the 8-SNP *MTR* haplotypes #1 and #11 (0.52 for AGCTAATT and 0.67 for AGCTACCT, respectively) and for three SNPs in *DNMT3A* when examined within the context of multivitamin use [0.54 for 3′ UTR C>G (rs13420827), 0.57 for intron 6 G>A (rs11887120), and 0.66 for intron 22 A>T (rs11695471)]. These calculations suggest that the probability of our findings being falsely positive is 9% to 16% for the *SHMT1* SNPs and higher for the other SNPs.

Discussion

To our knowledge, we are the first to examine a large number of SNPs (n = 180) in genes (n = 21) related to the 1-C transfer and methylation-related pathways for ovarian cancer risk among Caucasians in the United States, and the findings provide an initial report of potential causal variants, most of which are novel for their previously unexamined association with ovarian cancer. We extend findings of the recently reported association of *SHMT1* SNPs in other cancers and confirm their relevance to ovarian cancer. Interactions with multivitamin intake are suggestive as are haplotypes in *MTR*, but the absence of HWE could have resulted in spurious associations. No definitive differences were observed across histologic subtypes.

The vitamin B6–dependent SHMT1 enzyme catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylenetetrahydrofolate in the cytoplasm for the synthesis of methionine, thymidylate, and purines (43). Both the incorporation of the β-carbon of serine into DNA and SHMT1 activity are increased when cells are stimulated to proliferate (44). SHMT1 activity is also elevated in tumor tissues (45). In our study, the two haplotypes in *SHMT1* with significant risk associations differed only at a single locus, intron 5 A>G (rs9909104), which was also independently associated with ovarian cancer. We and others (20, 22, 23, 28, 41, 46, 47) observed generally null associations with *SHMT1* Leu435Phe (rs1979277) with various outcomes, and previous studies found no associations with *SHMT1* exon 12 C>T (rs1979276; refs. 13, 41, 47) and 3′ UTR C>G (rs3783; ref. 41). The two latter SNPs were not genotyped in our study but were tagged at pairwise $r^2 \geq 0.8$ by the assayed SNPs that compose the 5-SNP *SHMT1* haplotype [Leu435Phe, 3′ UTR T>C (rs12952556) and 3′ UTR G>A (rs921986); Fig. 1]. Although different yet correlated loci were examined, our findings are supported by those of Zhang et al. (41), who reported significantly altered risk of squamous cell carcinoma of the head and neck from haplotypes comprising three *SHMT1* SNPs [Leu435Phe, exon 12 C>T (rs1979276) and 3′ UTR C>G (rs3783)], and by the same group (40) of significantly altered risk of lung cancer associated with carrying an increased number of variant genotypes in five *SHMT1* SNPs [Leu435Phe, exon 12 C>T (rs1979276), 3′ UTR C>G (rs3783), promoter SNP C>A (rs643333), and promoter SNP G>C (rs638416)]. The observed associations from the same SNPs or SNPs that are highly correlated in each of our studies strongly suggest that these *SHMT1* SNPs may themselves be or are in strong LD with putative causal alleles. Mutations in the COOH-terminal region of *SHMT1* lead to incorrect protein folding (48) and the location of the SNPs near or in the 3′ UTR region of the gene suggests that they may affect enzyme conformation and activity. Fine-mapping of this chromosomal region with further association testing is therefore a recommended priority for future studies.

Suggestive findings were observed with SNPs in *DNMT3A*, particularly when stratified by multivitamin use. The DNMT3A enzyme functions principally in *de novo* methylation (49). In one of the few studies to examine *DNMT3A* SNPs for cancer risk, Cebrian et al. (14) did not find significant associations of 13 tagSNPs with breast cancer risk. Eight tagSNPs in that study were also genotyped in the present analysis or were represented in our data by tagged SNPs. None of these eight were the three SNPs in *DNMT3A* for which we observed significant associations by multivitamin supplement use. Our findings could be due to chance, or there may have been greater power to detect the gene effect among the homogeneous subset of the population defined by multivitamin intake. Because these analyses were secondary and comprised small numbers of subjects, confirmation of our findings is necessary.

Fifteen SNPs had genotype distributions significantly different from that expected under HWE; nine are expected by chance. We did not find obvious deviations among cluster plots and genotype calls and no evidence for statistical heterogeneity in genotype distributions between sites. Furthermore, Mayo and Duke subjects were genotyped in the same laboratory using the same platform with high genotyping concordance and similar call rates observed among cases and controls, suggesting that any genotyping errors (and therefore differences in HWE) were nondifferential. Deviations from HWE can be due to several reasons, including factors unrelated to genotyping error such as the presence of a deletion polymorphism or copy number variation (42). We conclude that the findings may be due to chance or random error, which would attenuate risk associations.

The strengths of our study include the large sample size and coordinated data collection across study sites, the investigation of a large number of SNPs across 21 genes with well-defined roles in 1-C transfer and methylation, and the robust genotyping and analytic protocols. The comparable associations of the *SHMT1* haplotype in this study and those by the Spitz investigative team for risk of other cancers (40, 41) merit further consideration and strengthen the utility of tagSNPs, bioinformatics tools, and haplotype analyses for identifying common genetic variants in disease risk. Although preliminary, the genes that interacted with multivitamin use in our study potentially support a nutrigenetic role (50) of 1-C donor units in pathways that influence both genetic expression via methylation (e.g., *DNMT*'s) and enzyme activity via disruption of transfer of 1-C units (e.g., *SHMT1*).

Some potential limitations of our study warrant discussion. First, this was not a comprehensive examination of 1-C metabolism genes. Second, different definitions and reference periods defined

Table 4. ORs and 95% CIs for the joint effect of selected polymorphisms and multivitamin supplement use for ovarian cancer risk among 1,770 Caucasian subjects, Mayo Clinic and Duke University, 1999–2006

Gene/SNP rsID	Multivitamin nonusers			
	Homozygous common allele (Referent)	Heterozygous	Homozygous rare allele	Ordinal (per rare allele)
	Cases/controls	Cases/controls	Cases/controls	OR (95% CI)
DNMT3A				
11887120	93/149	142/175	70/64	1.3 (1.0–1.6)
11695471	154/165	123/163	30/58	0.8 (0.6–1.0)
13420827	192/252	95/129	19/6	1.3 (1.0–1.7)
DNMT1				
9305012	268/345	36/42	4/1	1.3 (0.9–2.1)
MTHFR				
2184226	252/337	53/48	2/3	1.3 (0.9–1.9)
MTHFD1				
17101854	296/367	13/21	0	0.6 (0.3–1.3)

NOTE: ORs were age and state adjusted. Joint effect was determined using a log-additive logistic regression model.
*At least 4 pills/wk during the previous year (Mayo subjects) or ≥1 pill/wk during the past 5 y (Duke subjects).

regular multivitamin use between sites, complicating our measure of exposure time. We did not have information on dietary intake and could not verify which nutrient(s) was related to the modifying effects of multivitamins. In addition, 50% to 65% of Mayo and Duke controls reported taking multivitamins compared with 38% to 40% of women in the United States (51), and the greater prevalence among Mayo compared with Duke controls could be attributable to fewer smokers, higher level of education, and the older age of Mayo

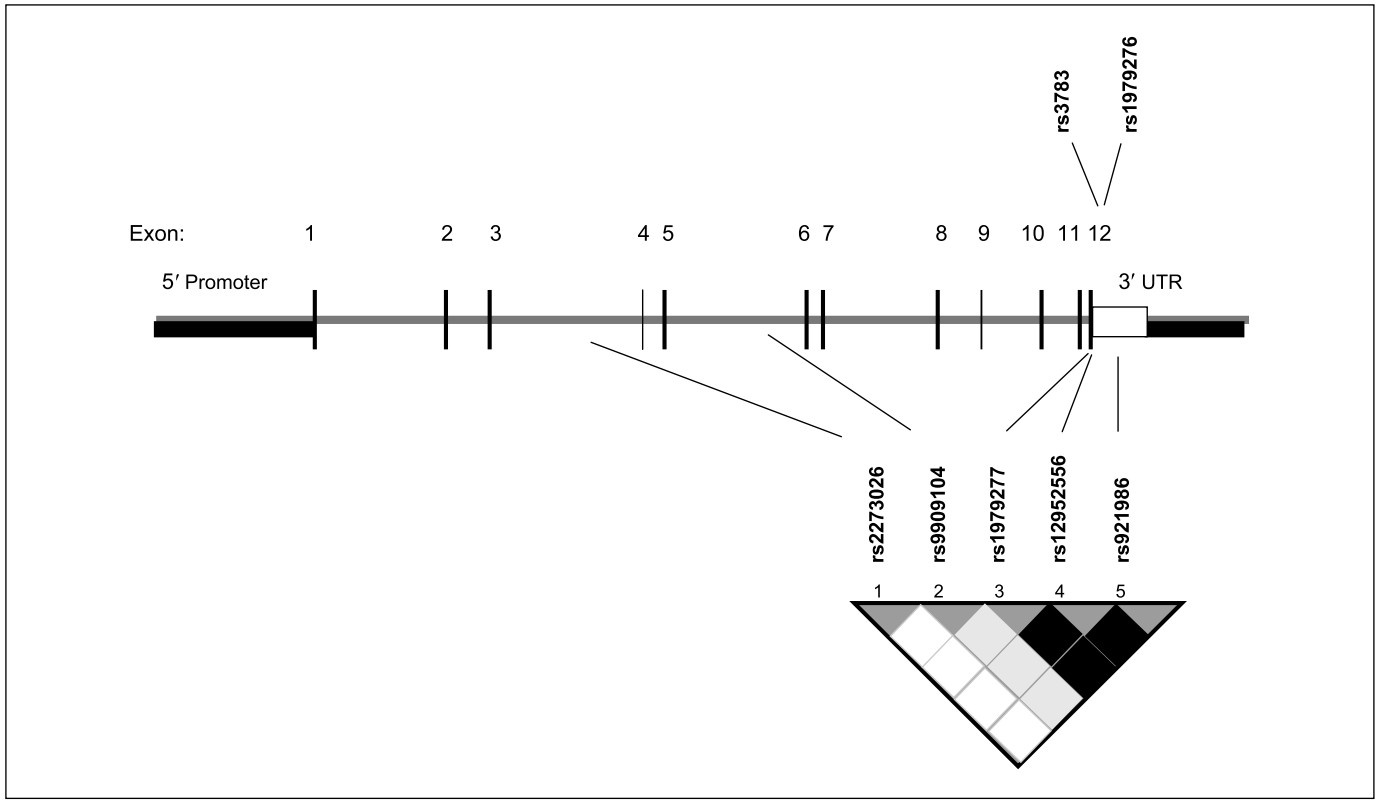

Figure 1. Gene structure and location of SNPs in the *SHMT1* gene. TagSNPs that compose the haplotype assayed among 1,770 Caucasian subjects (Mayo Clinic and Duke University, 1999–2006) are shown below the gene. SNPs previously reported to be associated with cancer (40, 41) in the same region are shown above the gene. Adapted from ref. 40. Shaded regions in the LD plot indicate the strength of LD between pairwise combinations of SNPs (*white,* low LD; *black,* high LD).

Table 4. ORs and 95% CIs for the joint effect of selected polymorphisms and multivitamin supplement use for ovarian cancer risk among 1,770 Caucasian subjects, Mayo Clinic and Duke University, 1999–2006 (Cont'd)

Multivitamin users*				$P_{interaction}$
Homozygous common allele (reference)	Heterozygous	Homozygous rare allele	Ordinal (per rare allele)	
Cases/controls	Cases/controls	Cases/controls	OR (95% CI)	
124/157	151/264	58/88	0.8 (0.7–1.0)	0.007
126/224	170/234	39/51	1.2 (1.0–1.5)	0.01
237/321	84/166	13/20	0.8 (0.6–1.0)	0.006
306/442	30/65	0/2	0.6 (0.4–1.0)	0.03
418/288	44/80	3/9	0.7 (0.5–1.1)	0.05
311/488	25/21	0	2.0 (1.0–3.7)	0.02

controls, which are factors associated with multivitamin use (51). Adjustment for these factors, however, did not alter risk estimates noticeably. Third, in our analyses of effect modification by multivitamin use, the sample was too small to examine genetic models other than log-additive where the relationship with ovarian cancer of the main effect of genotype seemed to deviate from an ordinal relationship.

In conclusion, our data provide evidence for genetic variation in *SHMT1* with ovarian cancer risk, as well as noteworthy associations with *DNMT3A* and *MTR*. Although the modifying effects of multivitamins as suppliers of 1-C units are suggestive, replication of these findings should be pursued by other investigators in other populations, including those with detailed information on diet.

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