Null Results in Brief

DNA Promoter Methylation in Breast Tumors: No Association with Genetic Polymorphisms in *MTHFR* and *MTR*

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Abstract

Aberrant promoter methylation is recognized as an important feature of breast carcinogenesis. We hypothesized that genetic variation of genes for methylenetetrahydrofolate reductase (*MTHFR*) and methionine synthase (*MTR*), two critical enzymes in the one-carbon metabolism, may alter DNA methylation levels and thus influence DNA methylation in breast cancer. We evaluated case-control association of *MTHFR* C677T, A1298C, and *MTR* A2756G polymorphisms for cases strata-defined by promoter methylation status for each of three genes, *E-cadherin*, *p16*, and *RAR-β₂* in breast cancer; in addition, we evaluated case-case comparisons of the likelihood of promoter methylation in relation to genotypes using a population-based case-control study conducted in Western New York State. Methylation was evaluated with real-time methylation-specific PCRs for 803 paraffin-embedded breast tumor tissues from women with primary, incident breast cancer. We applied unordered polytomous regression and unconditional logistic regression to derive adjusted odds ratios and 95% confidence intervals. We did not find any association of *MTHFR* and *MTR* polymorphisms with breast cancer risk stratified by methylation status nor between polymorphisms and likelihood of promoter methylation of any of the genes. There was no evidence of difference within strata defined by menopausal status, estrogen receptor status, folate intake, and lifetime alcohol consumption. Overall, we found no evidence that these common polymorphisms of the *MTHFR* and *MTR* genes are associated with promoter methylation of *E-cadherin*, *p16*, and *RAR-β₂* genes in breast cancer. (Cancer Epidemiol Biomarkers Prev 2009;18(3):998–1002)

Introduction

Both CpG island promoter hypermethylation and global DNA hypomethylation are prominent features of breast tumors and are important in the carcinogenic process (1, 2). However, the factors that result in aberrant DNA methylation in normal and neoplastic tissues are not well known. The one-carbon metabolism, critical in the availability of methyl groups and therefore DNA methylation, may affect both hypomethylation and hypermethylation (3). We examined whether common genetic variations of one-carbon metabolism genes, specifically methylenetetrahydrofolate reductase (*MTHFR*) and methionine synthase (*MTR*), are associated with promoter methylation of the three genes selected because they are functionally important and have been found to be methylated in breast tumors and may therefore influence breast carcinogenesis: *E-cadherin*, involved in cell adhesion (4); *p16*, important in cell cycle regulation (5); and retinoic acid binding receptor-β₂ (*RAR-β₂*), important in receptor-mediated cell signaling (6). These associations were examined in a case-control study of primary, incident breast cancers, examining the association between *MTHFR* and *MTR* polymorphisms and breast cancer risk by tumor methylation status as well as the likelihood of methylation in tumors by genotype.

Materials and Methods

Detailed study methods have been published previously (7). In brief, the Western New York Exposures and Breast Cancer Study included 1,170 primary, histologically confirmed, incident breast cancer cases, ages 35 to 79 at diagnosis and 2,115 randomly selected population controls, frequency-matched to cases on age and race. Extensive in-person interviews and self-administered questionnaires were administered to participants including queries on demographic factors and breast cancer risk factors. The response rates were 72% for cases and 63% for controls. Information on tumor size, histologic

grade, and cancer stage was abstracted from medical charts using a standard protocol. Estrogen receptor (ER) status was determined by immunohistochemistry as described previously (7). DNA was extracted from blood and mouthwash samples using the GenQuik DNA Extraction Kit (BioServe Biotechnologies, Ltd.). Archived tumor blocks were obtained from 920 (78.6%) breast cancer cases.

The allelic discrimination of the *MTHFR* C677T, A1298C, and *MTR* A2756G polymorphisms were assessed by real-time PCR with TaqMan genotyping assay with primers, probes, and conditions as described on the National Cancer Institute's Cancer Genome Anatomy Project SNP500 Cancer Database web site.[6] Promoter methylation of *E-cadherin*, *p16*, and *RAR-β_2* was determined by real-time methylation-specific PCR after bisulfite modification of genomic tumor DNA isolated from archived paraffin-embedded tissues of 803 breast cancer tumors (described in detailed previously; ref. 7).

The exact χ^2 goodness-of-fit test was used to test the Hardy-Weinberg equilibrium of the genotypes. Characteristics of participating cases with and without promoter methylation of specific genes and controls were compared using the ANOVA test for continuous variables and χ^2 test for categorical variables. For comparisons of cases with and without promoter methylation to controls, odds ratios (OR) and 95% confidence intervals (CI) were calculated using polytomous logistic regression. Unconditional logistic regression was used for case-case comparisons of those with and without promoter methylation to estimate the ORs and 95% CIs for the associations of *MTHFR* and *MTR* genotypes with promoter methylation in breast cancer. Interactions between genotype and menopause, ER status, folate intake, or alcohol intake were evaluated by evaluation of a multiplicative term in the regression model. All analyses were adjusted by age and race. For case-case comparisons, we also adjusted for ER status. Potential confounding effects of other demographic factors and known breast cancer risk factors were also examined, and the results changed by <10% (data not shown). All statistical tests were based on two-sided probability. All statistical analyses were done using SAS version 9.1 (SAS Institute).

Results

The frequencies of genotypes of *MTR* and *MTHFR* polymorphisms for cases and controls and distributions of selected characteristic factors are shown in Table 1. All polymorphisms were in Hardy-Weinberg equilibrium for cases and controls in both the whole population and in Caucasians only. The distributions of *MTR* and *MTHFR* polymorphisms were similar for cases with or without *E-cadherin*, *p16*, and *RAR-β_2* gene promoter methylation.

The results of case-case and case-control comparisons evaluating the associations between *MTR* A2756G and *MTHFR* C677T and C1298A genotypes and breast tumors with or without promoter methylation are pre-

sented in Table 2. In case-control comparisons, we did not find any association between *MTHFR* and *MTR* polymorphisms with breast cancer risk stratified by methylation status. In case-case analyses, there was no association between *MTR* A2756G, *MTHFR* C677T, or C1298A polymorphisms and *E-cadherin*, *p16*, or *RAR-β_2* gene methylation in breast tumors for both premenopausal and postmenopausal women. We also analyzed the above relations using dominant and recessive models of inheritance in both case-control comparisons and case-case comparisons, however, no associations were observed. In addition, results of analyses stratified by ER status, folate intake, and lifetime alcohol intake were similar; there were no interactions and all values for interaction tests were $P > 0.05$. For the joint effects of *MTR* and *MTHFR* genotypes on the likelihood of promoter methylation in at least one gene, compared with the *MTR* 2756AA, *MTHFR* 677CC, and 1298AA genotype, women with breast cancer with more variant alleles for either *MTR* 2756G, *MTHFR* 677T, or 1298C alleles tend to increase the likelihood of promoter methylation in at least one gene, although associations were not statistically significant and there was no trend (ORs, 1.21, 1.30, and 1.45; 95% CIs, 0.65-2.22, 0.70-2.41, and 0.72-2.91 for women with any one, any two, and three variant alleles, respectively).

Discussion

MTHFR and MTR are key enzymes in the biosynthesis of 5-methyl tetrahydrofolate and methionine, which are precursors for DNA methylation reactions; and these enzymes' activities also affect the availability of tetrahydrofolate for nucleotide biosynthesis (3). *MTHFR* C677T and A1298C variants are associated with a reduction of enzyme activity (8, 9) and the *MTR* A2756G variant is associated with lower homocysteine concentrations (10, 11), and have been investigated for their possible effect on breast carcinogenesis with inconsistent results (12-17).

Polymorphisms of *MTHFR* C677T and A1298C and *MTR* A2756G have been investigated in relation to promoter methylation of genes in breast tumors in one other study of 227 breast cancer cases. Consistent with our findings, no association was observed between *MTHFR* C677T and *MTR* A2756G polymorphisms and the frequency of promoter methylation in seven genes, including *E-cadherin*, *p16*, and *RAR-β_2*, in breast cancer (18). In our study, we also found that this association was not modified by menopausal status, ER status, folate intake, or total alcohol intake.

There have been studies examining these genotypes with promoter methylation of genes in tumors from other sites with inconsistent results (19-26). Curtin et al. found an increased likelihood of highly CpG-methylated phenotype in colon tumors for those with one or two variant *MTHFR* 1298C alleles, and the association was modified by high-risk dietary profiles (low folate and methionine intake and high alcohol use; ref. 19). In other studies, these genotypes were not associated with the likelihood of *p16* promoter hypermethylation of colorectal cancers (20-22). Similarly, *MTHFR* C677T, A1298C, or *MTR* C2756G genotypes were not associated with *E-cadherin* and *p16* promoter methylation in esophageal (23) and cervical cancers (25).

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[6] http://snp500cancer.nci.nih.gov

Table 1. Distribution of one-carbon metabolism genes among breast cancer cases, cases with (M) and without (UM) promoter methylation and controls (Western New York Exposures and Breast Cancer Study, 1996-2001)

	Controls	E-cadherin		p16		RAR-β_2	
		M (n = 161)*	UM*	M (n = 208)*	UM*	M (n = 221)*	UM*
Age, y[†]	57.8 ± 11.8	58.0 ± 11.8	57.4 ± 11.2	58.0 ± 11.2	57.4 ± 11.3	57.4 ± 11.3	57.6 ± 11.3
Race/ethnicity							
White	1,910 (90.3%)	148 (91.9%)	594 (92.5%)	192 (92.3%)	550 (92.4%)	210 (95.0%)	532 (91.4%)
Non-white	205 (9.7%)	13 (8.1%)	48 (7.5%)	16 (7.7%)	45 (7.6%)	11 (5.0%)	50 (8.6%)
Postmenopausal	1,503 (71.1%)	111 (68.9%)	455 (70.9%)	147 (70.7%)	419 (70.4%)	157 (71.0%)	409 (70.3%)
Folate intake (mg/d)[†,‡]	267.0 ± 147.3	281.7 ± 178.4	280.7 ± 178.4[§]	275.2 ± 143.2	282.9 ± 158.9[§]	285.5 ± 175.0	279.2 ± 146.6
Lifetime alcohol (oz)[†,‖]	3,545.6 ± 12,546.9	3,509.3 ± 6,278.0	2,901.8 ± 4,766.5	3,049.5 ± 5,194.3	3,023.3 ± 5,104.7	2,626.4 ± 3,703.2	3,177.4 ± 5,548.6
ER status							
+		114 (71.3%)	446 (70.0%)	136 (65.7%)	424 (71.9%)	163 (74.8%)	397 (68.6%)
−		46 (28.7%)	191 (30%)	71 (34.3%)	166 (28.1%)	55 (25.2%)	182 (31.4%)
MTR (A2756C)							
AA	1,217 (63.1%)	99 (65.1%)	392 (65.0%)	128 (65.7%)	363 (64.8%)	130 (63.1%)	361 (65.8%)
AG	619 (32.1%)	47 (30.9%)	179 (29.7%)	56 (28.7%)	170 (30.4%)	66 (32.0%)	160 (29.1%)
GG	93 (4.8%)	6 (4.0%)	32 (5.3%)	11 (5.6%)	27 (4.8%)	10 (4.9%)	28 (5.1%)
χ^2 test, P value			0.78		0.84		0.74
MTHFR (A1298C)							
AA	864 (47.2%)	60 (44.8%)	249 (47.2%)	80 (46.3%)	229 (46.8%)	79 (43.4%)	230 (47.9%)
AC	779 (42.5%)	63 (47.0%)	224 (42.4%)	76 (43.9%)	211 (43.2%)	88 (48.4%)	199 (41.5%)
CC	188 (10.3%)	11 (8.2%)	55 (40.4%)	17 (9.8%)	49 (10.0%)	15 (8.2%)	51 (10.6%)
χ^2 test, P value			0.56		0.98		0.25
MTHFR (C677T)							
CC	813 (43.9%)	63 (44.7%)	260 (45.1%)	83 (44.1%)	240 (45.4%)	93 (47.4%)	230 (44.2%)
CT	816 (44.1%)	57 (40.4%)	248 (43.1%)	74 (39.4%)	231 (43.7%)	87 (44.4%)	218 (41.8%)
TT	223 (12.0%)	21 (14.9%)	68 (11.8%)	31 (16.5%)	58 (10.9%)	16 (8.2%)	73 (14.0%)
χ^2 test, P value			0.59		0.13		0.11

NOTE: Subjects with missing values were excluded from the analysis.

*M, methylated; UM, unmethylated.

[†]Mean ± SD.

[‡]Dietary folate intake.

[§]Comparison of unmethylated cases to controls ($P < 0.05$).

[‖]Among ever drinkers.

Table 2. Associations of *MTR* and *MTHFR* genotypes and gene promoter methylation: ORs (95% CIs) for comparisons of controls to cases with promoter methylation (M), to cases without methylation (UM), and case-case comparisons

	MTR A2756G (rs1805087)			*MTHFR* A1298C (rs1801131)			*MTHFR* C677T (rs1801133)		
	AA	AG	GG	AA	AC	CC	CC	CT	TT
Premenopausal									
E-cadherin									
M/U/control	30/121/370	15/44/161	3/6/31	19/73/257	15/53/233	4/18/49	22/73/227	15/68/251	5/21/68
M vs. controls*	1.0	1.22 (0.64-2.31)	1.22 (0.35-4.21)	1.0	0.71 (0.37-1.35)	0.89 (0.30-2.64)	1.0	0.59 (0.31-1.13)	0.71 (0.26-1.90)
U vs. controls*	1.0	0.83 (0.57-1.23)	0.57 (0.23-1.40)	1.0	0.67 (0.46-0.97)	1.07 (0.60-1.92)	1.0	0.82 (0.58-1.18)	0.92 (0.53-1.59)
M vs. U†	1.0	1.49 (0.74-3.02)	2.24 (0.51-9.88)	1.0	1.05 (0.52-2.12)	0.62 (0.17-2.26)	1.0	0.73 (0.36-1.48)	0.78 (0.27-2.29)
p16									
M/U/control	42/109/370	44/15/161	1/8/31	27/65/257	19/49/233	5/17/49	24/71/227	24/59/251	8/18/68
M vs. controls*	1.0	0.87 (0.47-1.60)	0.28 (0.04-2.13)	1.0	0.77 (0.43-1.38)	0.95 (0.36-2.56)	1.0	1.01 (0.57-1.79)	1.21 (0.52-2.79)
U vs. controls*	1.0	0.92 (0.62-1.36)	0.84 (0.38-1.88)	1.0	0.65 (0.44-0.94)	1.06 (0.58-1.92)	1.0	0.70 (0.49-1.01)	0.77 (0.44-1.37)
M vs. U†	1.0	0.93 (0.47-1.83)	0.31 (0.04-2.61)	1.0	1.16 (0.61-2.24)	0.96 (0.32-2.84)	1.0	1.45 (0.76-2.78)	1.55 (0.60-4.00)
RAR-β2									
M/U/control	37/114/370	22/37/161	1/8/31	25/67/257	16/52/233	6/16/49	25/70/227	25/58/251	5/21/68
M vs. controls*	1.0	1.39 (0.80-2.42)	0.33 (0.04-2.49)	1.0	0.52 (0.28-0.95)	0.91 (0.36-2.25)	1.0	0.81 (0.47-1.41)	0.58 (0.22-1.55)
U vs. controls*	1.0	0.75 (0.50-1.13)	0.80 (0.36-1.80)	1.0	0.74 (0.51-1.09)	1.09 (0.59-2.00)	1.0	0.75 (0.52-1.09)	0.99 (0.57-1.71)
M vs. U†	1.0	1.86 (0.97-3.55)	0.45 (0.05-3.92)	1.0	0.76 (0.39-1.50)	0.96 (0.34-2.69)	1.0	1.04 (0.55-1.94)	0.56 (0.19-1.63)
Postmenopausal									
E-cadherin									
M/U/control	69/271/847	32/135/458	3/26/62	41/176/607	48/171/546	7/37/139	42/187/586	42/180/565	16/47/155
M vs. controls*	1.0	0.91 (0.59-1.39)	0.66 (0.23-2.15)	1.0	1.24 (0.83-1.86)	0.72 (0.32-1.62)	1.0	1.07 (0.70-1.63)	1.46 (0.81-2.63)
U vs. controls*	1.0	1.00 (0.79-1.26)	1.47 (0.91-2.38)	1.0	0.99 (0.79-1.24)	0.85 (0.57-1.26)	1.0	1.04 (0.83-1.31)	0.98 (0.69-1.41)
M vs. U†	1.0	0.89 (0.56-1.41)	0.43 (0.13-1.49)	1.0	1.20 (0.78-1.86)	0.84 (0.36-2.00)	1.0	1.02 (0.65-1.62)	1.50 (0.78-2.88)
p16									
M/U/control	86/254/847	41/126/458	10/19/62	53/164/607	57/162/546	12/32/139	59/169/586	50/172/565	23/40/155
M vs. controls*	1.0	0.92 (0.63-1.36)	1.76 (0.57-3.56)	1.0	1.04 (0.73-1.50)	0.87 (0.46-1.65)	1.0	0.89 (0.61-1.30)	1.47 (0.89-2.43)
U vs. controls*	1.0	1.00 (0.78-1.27)	0.76 (0.87-3.56)	1.0	1.04 (0.82-1.31)	0.81 (0.53-1.22)	1.0	1.10 (0.88-1.39)	0.93 (0.61-1.36)
M vs. U†	1.0	0.90 (0.59-1.38)	1.55 (0.69-3.50)	1.0	0.97 (0.65-1.45)	1.08 (0.53-2.22)	1.0	0.82 (0.54-1.26)	1.61 (0.89-2.89)
RAR-β2									
M/U/control	93/247/847	44/123/458	9/20/62	54/163/607	72/147/546	9/35/139	68/160/586	62/160/565	11/52/155
M vs. controls*	1.0	0.92 (0.64-1.33)	1.46 (0.70-3.03)	1.0	1.35 (0.94-1.91)	0.67 (0.33-1.37)	1.0	0.96 (0.68-1.37)	0.61 (0.43-0.88)
U vs. controls*	1.0	1.00 (0.79-1.27)	1.25 (0.74-2.11)	1.0	0.93 (0.74-1.18)	0.88 (0.59-1.31)	1.0	1.08 (0.85-1.38)	1.27 (0.66-2.45)
M vs. U†	1.0	0.90 (0.60-1.37)	1.20 (0.52-2.75)	1.0	1.45 (0.98-2.13)	0.68 (0.30-1.54)	1.0	0.87 (0.58-1.29)	0.48 (0.24-0.97)

*ORs and 95% CIs adjusted for age and race.
†ORs and 95% CIs also adjusted for ER status.

The strengths of our study include the population-based study design and a relatively large sample size, leading to more stable risk estimates. Nevertheless, the number of cases in subgroups were small and the CIs were wide. We had 80% power to detect ORs of 2.5 for the association between *MTR* genotype and methylation and an OR of 2.0 for the association between *MTHFR* polymorphisms and methylation. Additionally, the study applied the candidate gene approach and primarily focused on potential common genetic variants (>5%) and polymorphisms with amino acid changes. We cannot rule out the possibility that genetic polymorphisms of one-carbon metabolisms other than those included in our study may be related to the likelihood of methylation. Given multiple genes for proteins involved in one-carbon metabolism (3), the confounding and/or modifying effects of other genes also cannot be excluded. Further lack of response among cases has potential for selection bias. Compared with participating cases, those non-participants were of a somewhat lower education level and older. However, the two groups were similar in terms of tumor stage, distant metastases, and other breast cancer risk factors. Furthermore, our inability to obtain the paraffin-embedded breast tumor tissue for all cases may have led to bias. In comparisons of cases without available archived tumor tissue to those with available tissue, those with tissue were somewhat younger at diagnosis and had a higher tumor-node-metastasis stage of breast tumor. However, the two groups were similar in terms of tumor size, histologic grade, nuclear grade, ER and progesterone receptor status. With regard to the validity of the measure of methylation in formalin-fixed paraffin-embedded tissue, a recent study showed a high correlation in methylation between paraffin-embedded tumor tissues and fresh samples from the same subject, measured with a similar real-time PCR method as ours, concluding that paraffin-embedded samples are well suited for methylation assessment (27).

In summary, we found no evidence that these common polymorphisms of the *MTHFR* and *MTR* genes were associated with the prevalence of promoter methylation of *E-cadherin*, *p16*, and *RAR-β₂* genes in breast tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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BLOOD CANCER DISCOVERY

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