Methionine-Dependence Phenotype in the *de novo* Pathway in BRCA1 and BRCA2 Mutation Carriers with and without Breast Cancer

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Abstract

Methionine-dependence phenotype (MDP) refers to the reduced ability of cells to proliferate when methionine is restricted and/or replaced by its immediate precursor homocysteine. MDP is a characteristic of human tumors *in vivo*, human tumor cell lines, and normal somatic tissue in some individuals. It was hypothesized that MDP is a risk factor for developing breast cancer in BRCA (*BRCA1* and *BRCA2*) germline mutation carriers. To test the hypothesis, human peripheral blood lymphocytes of BRCA carriers with and without breast cancer and healthy non-carrier relatives (controls) were cultured for 9 days in medium containing either 0.1 mmol/L L-methionine or 0.2 mmol/L D,L-homocysteine, with the ratio of viable cell growth in both types of medium after 9 days used to calculate the methionine-dependence index (MDI), a measure of MDP. We also tested whether MDP was associated with common polymorphisms in methionine metabolism. Viable cell growth, MDI, and polymorphism frequency in *MTRR* (A66G and C524T) and *MTHFR* (A1298C and A1793G) did not differ among the study groups; however, MDI tended to be higher in BRCA carriers with breast cancer than those without and was significantly increased in *MTHFR* 677T allele carriers relative to wild-type carriers ($P = 0.017$). The presence of *MTR* A2756G mutant allele and *MTHFR* C677T mutant allele in carriers was associated with increased breast cancer risk [odds ration, 3.2 ($P = 0.16$; 95% confidence interval, 0.76-13.9) and 3.9 ($P = 0.09$; 95% confidence interval, 0.93-16.3), respectively]. The results of this study support the hypothesis that defects in methionine metabolism may be associated with breast cancer risk in BRCA carriers. (Cancer Epidemiol Biomarkers Prev 2008;17(10):2565–71)

Introduction

Methionine is an amino acid, which can be obtained exogenously via the diet and/or endogenously, by either methylation of homocysteine or transamination of 4-methylthio-2-oxobutanoic acid, through the *de novo* and salvage pathways, respectively (1). Evidence from two studies suggests that the majority of methionine produced through the salvage pathway is transformed to S-adenosylmethionine (SAM), whereas methionine synthesized through the *de novo* pathway by homocysteine methylation is predominantly used for protein synthesis (2, 3). SAM, in its turn, is required for the formation of spermidine and spermine, which have been suggested to affect cell proliferation (4), and is the primary methyl donor for DNA methylation, which regulates gene expression.

Methionine dependency is the term used to describe the phenotype of decreased ability of cells to proliferate when methionine is replaced by one of its immediate precursors, such as homocysteine, when other methyl donors, such as folate, vitamin B_{12}, and choline are present in adequate amounts. Methionine-dependency phenotype (MDP) has been frequently observed in human tumor cell lines (5, 6); however, human lymphocytes and lymphoid cell lines have also been shown to exhibit varying degrees of methionine-dependency (7, 8). When methionine is replaced by homocysteine in the culture medium, methionine-dependent cells exhibit decreased nuclear division and/or cell growth, reduced levels of SAM, and increased levels of S-adenosylhomocysteine compared with methionine-independent cells (6-9). Concomitantly, a reduced SAM/S-adenosylhomocysteine ratio induces global DNA hypomethylation and specific gene promoter CpG islands hypermethylation, both features of cancer cells that can alter chromatin structure and gene expression and thought to precede mutational and chromosomal abnormalities that are involved in cancer progression (10, 11). A study of methionine dependence in skin fibroblasts of patients with hereditary colon cancer showed that the MDP was expressed in normal somatic tissue several years before the clinical manifestation of cancer, suggesting that the MDP in these cells was an inherited trait possibly related to increased risk of oncogenic transformation and/or tumor growth (12). MDP has been observed in both breast cancer cell lines and tumors (5, 6).

Received 2/13/08; revised 7/9/08; accepted 7/14/08.

Grant support: Cecilia Kilkeary Foundation.

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doi:10.1158/1055-9965.EPI-08-0140

BRCA1 (13) and *BRCA2* (14) germline mutations are linked to 5% to 10% of all breast cancer cases diagnosed. *BRCA1* and *BRCA2* code for proteins of importance to genome stability as both have been shown to be involved in several cellular mechanisms such as homologous recombinational repair of DNA strand breaks, transcriptional regulation, cell cycle control, and/or mitotic spindle formation (15, 16); however, not all *BRCA1* and *BRCA2* mutation carriers develop breast cancer (~85% of women carrying a *BRCA1* or *BRCA2* mutation will develop breast cancer by age 70 years) and other inherited traits may modify their cancer risk (17). Therefore, the primary aim of this study was to test the hypothesis that MDP in peripheral blood lymphocytes may be an inherited or acquired trait associated with an increased risk for developing breast cancer in *BRCA1* and *BRCA2* germline mutation carriers. A secondary aim of the study was to obtain preliminary data on the possible influence of common polymorphisms in the methionine *de novo* synthesis pathway in genes such as *MTHFR* (C677T, A1298C, and A1793G), *MTR* (A2756G), and *MTRR* (A66G and C524T) on MDP and breast cancer risk. MTHFR converts 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate, the methyl donor required for methionine synthesis by methylation of homocysteine; MTR is the vitamin B_{12}-dependent enzyme required for the synthesis of methionine from homocysteine, and 5-methyl and MTRR function is required to maintain vitamin B_{12} in its reduced active form. Homozygosity for the C677T or A1298C mutation of the gene encoding *MTHFR* causes a reduction in its enzyme activity, resulting in limited methyl-group availability for the conversion of homocysteine to methionine (18, 19), which subsequently may affect exogenous methionine requirement. It is not known whether a mutation in A1793G of the gene encoding MTHFR or in any of the previously mentioned mutations of *MTRR* (A66G and C524T) and *MTR* (A2756G) significantly alters capacity to generate methionine from homocysteine.

Materials and Methods

Approval for this study was obtained from the Human Experimental Ethics committees of Commonwealth Scientific and Industrial Research Organisation Health Sciences and Nutrition, University of South Australia and Children's, Youth and Women's Health Service in Adelaide, South Australia.

Subjects were recruited by mail from a database of breast cancer families who had previously undergone *BRCA1* or *BRCA2* gene mutation testing at Familial Cancer Unit of the South Australian Clinical Genetics Service, which provides a state-wide program for the population of 1.5 million and is the only such provider of genetic counseling and testing for familial cancer in South Australia. The women were referred to the Familial Cancer Unit because of a personal and familial history suggestive of familial breast cancer. Testing of the *BRCA1* and *BRCA2* genes was offered to women who fell within the ''high genetic risk'' category as defined by the National Health and Medical Research Council (20). Once a mutation has been identified in an affected person, genetic counseling and testing is offered to first- and second-degree relatives of the know carrier in the family, obligate carriers in the family, and presumed carriers in the family. In practice, ~40% of the at-risk relatives identified by the South Australian Clinical Genetics Service seek genetic counseling and testing (21). We have not identified any characteristics, which distinguish who seek genetic counseling and testing versus those who do not. In total, 140 female BRCA carriers and noncarriers, ages >18 y and residing in metropolitan South Australia, were approached, on the basis that they had had presymptomatic genetic testing at the Familial Cancer Unit after the identification of an unequivocally pathogenic mutation in a family member. In addition, noncarriers were only approached if they were unaffected by any cancer of any type. We only approached those who lived in metropolitan South Australia because samples had to be processed within 4 h of collection. Sixty-six female subjects gave consent to be part of the study and were recruited into the following groups: (*a*) controls (n = 24), (*b*) mutation carriers without breast cancer (n = 20), and (*c*) mutation carriers with breast cancer (n = 22).

The average age of those consenting to be part of the study was 52 y, whereas the average age of those who declined to be part of the study was 50 y. In addition, among those consenting, the proband's (i.e., affected person) Manchester score (a measurement to predict the likelihood of identifying a *BRCA1* or *BRCA2* mutation; ref. 22) was 49, whereas the score was 43 among those who denied participation in the study. Testing is offered if the Manchester score is 15 or more, indicating that all study participants came from ''high-density'' breast cancer kindred. There was no evidence of systematic bias in the subject groups.

Controls consisted of noncarrier relatives of *BRCA1* or *BRCA2* mutation carriers. Before their presymptomatic genetic test, all members of the family were at 25% to 50% risk of inheriting the family's mutation. Once testing was completed and they were shown to not carry the family's mutation, they were categorized as being noncarrier controls for the purpose of this study.

At the time of the study, all *BRCA1* and *BRCA2* mutations in the carriers were considered pathogenic and to place a woman at high risk of developing breast cancer. The description and distribution of the *BRCA1* and *BRCA2* mutations among carriers with and without breast cancer have been published previously (23). Before presymptomatic testing, individuals not affected by breast cancer were at 25% to 50% risk of having the pathogenic mutation, which had been documented in another relative. They were only tested for that mutation and were not screened for mutations elsewhere in the *BRCA1* or *BRCA2* genes. The frequency of BRCA mutations in the general population is low (~1:1,000); therefore, screening for other BRCA mutations was not pursued in non-carriers of the family's mutation.

All participants with breast cancer completed chemotherapy and/or radiotherapy >6 mo before blood sample collection with the exception of one participant who completed treatment one month before blood collection.

Methionine-Dependence Assay. To test the MDP hypothesis peripheral blood lymphocytes from *BRCA1* and *BRCA2* germ line mutation carriers, with or without breast cancer, and noncarrier relatives free of cancer were cultured for 9 d in medium containing

either methionine or homocysteine and their relative growth under these conditions was measured. Lymphocytes were isolated from heparinized blood samples using Ficoll-Paque gradients (Amersham Biosciences) and stored in liquid nitrogen in cryovials containing a solution consisting of 90% fetal bovine serum (ThermoFisher) and 10% DMSO (Sigma). Cryovials were removed from the liquid nitrogen and thawed at 37°C, upon commencement of the experiments. When thawed, lymphocytes were washed twice in RPMI 1640 without L-glutamine, L-cystine, and L-methionine (Sigma) to which the following supplements were added 0.25 mmol/L L-cystine/L (Sigma), 2 mmol/L L-glutamine (Sigma), 1 mmol/L sodium pyruvate (ThermoFisher), 10% fetal bovine serum (Sigma), and 1% penicillin (5,000 IU/mL)/streptomycin (5 mg/mL) solution (ThermoFisher). Cells were then resuspended in 1.0 mL of medium, and cell number and viability were determined using a Coulter Counter (Coulter Electronics) and Trypan Blue (Sigma) exclusion, respectively, to calculate the volume of cell suspension required to set up the cultures at 0.5×10^6 viable cells/mL. For each volunteer, 2 cultures were set up in 10 mL sterile conical tubes (Technoplas) for both Met^+Hcy^- and Met^-Hcy^+ conditions, containing 0.5×10^6 10^6 viable cells in 900 μL medium each. Cultures were then added to 100 μL of medium containing either 1 mmol/L L-methionine (Sigma) or 2 mmol/L D,L-homocysteine (Sigma), to achieve final culture concentrations of 0.1 mmol/L L-methionine (Met^+Hcy^-) or 0.2 mmol/L D,L-homocysteine (Met^-Hcy^+), respectively. Cells were stimulated to divide by adding phytohemagglutinin (30 μg/mL; Murex Biotech) and cultured for 9 d at 37°C in a humidified incubator with 5% CO_2. From day 1 until day 8, 100 μL medium was removed from the cultures and replaced by 100 μL of the appropriate medium containing 2 units of Interleukin-2 (Roche Diagnostics), every 24 h. On day 7 and day 9, cell number and viability were assessed using a Coulter Counter and trypan blue exclusion to determine the viable cell number in both types of medium. Viable cell number on day 9 was used to calculate the Methionine-Dependence Index (MDI) by dividing viable cell number in Met^+Hcy^- by viable cell number in Met^-Hcy^+ medium. The reproducibility of duplicate MDI measurements was determined by estimating the coefficient of variation, which was 15.8% ± 1.3% (mean ± SE for 64 separate assays done in duplicate). A high MDI indicates a decreased ability of the cells to grow in the absence of methionine and therefore an increased methionine dependence, whereas cells with a low MDI are less dependent on exogenous supply of methionine.

Genotyping for Polymorphism in the Methionine Pathway. DNA was isolated from lysed blood cells followed by Proteinase K (Sigma) treatment, salt extraction, and ethanol precipitation. *MTHFR* C677T, *MTHFR* A1298C, *MTHFR* A1793G, and *MTR* A2756G mutations were determined by PCR as described by Frosst et al. (19), Weisberg et al. (24), Wakutani et al. (25), and Van der Put et al. (26), respectively, whereas the *MTRR* A66G and *MTRR* C524T mutations were detected by PCR as reported by Janosíková et al. (27). Primers and restriction endonucleases were obtained from GeneWorks and New England Biolabs, respectively, whereas the deoxynucleotide triphosphates and Taq polymerase were acquired from Roche Diagnostics. Genotyping was done in duplicate to confirm validity of results. If the duplicates were not concordant, the test was repeated until a clear consistent result was obtained. Only 2% of duplicates were not concordant.

Statistical Analyses. Two-tailed Pearson correlations were used to analyze the potential confounding effect of age and/or body mass index (BMI) on viable cell growth and MDI. One-way ANOVA, followed by Tukey's *post hoc* multiple comparison test, was used to compare the age, BMI, and MDI of the controls, carriers without breast cancer and carriers with breast cancer, and the MDI in relation to selected polymorphisms in folate metabolism genes. In addition, viable cell number in both types of medium were compared using analysis of covariance (ANCOVA), with age and BMI included as covariates to control for their potential confounding effect. Assumptions relating to ANOVA and ANCOVA (i.e., normal distribution and homogeneity of variance) were examined and met. The paired *t* test (two tailed) was used to compare the effect of methionine replacement by homocysteine on viable cell number, and the χ^2 test was used to determine a possible association between genotype and *BRCA1* and *BRCA2* carrier and breast cancer status. Fisher Exact test was used to calculate odds ratios based on allele frequency. All data are expressed as mean ± SE. Significance was accepted at a P value of <0.05. All statistical analyses were done using SPSS 14.0 (SPSS). The choice of statistical tests was based on published recommendations (28).

Results

Age and BMI of Study Participants. The mean age of the 3 study groups (56.4 ± 2.44 years, 47.0 ± 3.12 years, and 51.0 ± 2.37 years for controls, BRCA carriers without breast cancer, and BRCA carriers with breast cancer, respectively) and BMI (27.6 ± 0.85 kg/m^2, 26.7 ± 0.92 kg/m^2, and 29.0 ± 1.32 kg/m^2 for controls, BRCA carriers without breast cancer, and BRCA carriers with breast cancer, respectively) did not differ significantly from each other, except for the age of the controls, which was significantly increased relative to BRCA carriers without breast cancer ($P = 0.045$).

Plasma Folate, Vitamin B_{12}, and Homocysteine of Study Participants. Plasma folate (nmol/L) in non-carrier controls, BRCA carriers without breast cancer, and BRCA carriers with breast cancer was 12.6 ± 0.5, 11.8 ± 0.8, and 12.4 ± 0.9, respectively, plasma vitamin B_{12} (pmol/L) was 295 ± 54, 345 ± 64, and 249 ± 17, respectively, and plasma homocysteine (μmol/L) was 7.7 ± 0.5, 7.1 ± 0.4, and 7.9 ± 0.3, respectively. The study groups did not differ significantly.

Viable Cell Number and MDI. Results in Fig. 1 show that (*a*) viable cell growth of peripheral blood lymphocytes is greater in medium containing Met^+Hcy^- relative to Met^-Hcy^+ ($P < 0.001$), and (*b*) growth was still on the increase on day 9 relative to day 7. These patterns of cell growth were also observed in preliminary experiments (data not shown), which also showed no evidence of viable cell growth in the absence of both methionine and homocysteine.

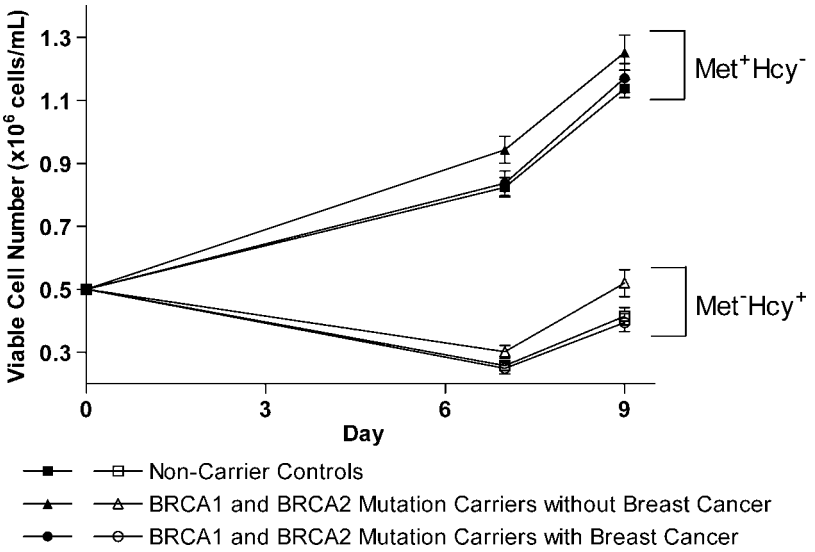

Figure 1. Viable cell number of *BRCA1* and *BRCA2* germline mutation carriers with or without breast cancer and noncarrier controls. Points, mean; bars, SE. For all study groups, viable cell number on day 9 was significantly increased relative to day 7 ($P < 0.001$), and viable cell number in $Met^{+}Hcy^{-}$ was significantly increased compared with $Met^{-}Hcy^{+}$ ($P < 0.001$). Viable cell number did not significantly differ between study groups on any given day and in either type of medium.

Viable cell number on day 9 was significantly negatively correlated with age and BMI (Table 1). Consequently, analyses were done using ANCOVA with age and BMI as covariates to control for their potential confounding effect. Viable cell number in both types of medium did not differ significantly between *BRCA1* and *BRCA2* mutation carriers with or without breast cancer and noncarrier controls on day 9 (Table 2). In addition, *BRCA1* and *BRCA2* carriers with or without breast cancer and noncarriers controls displayed a significantly increased viable cell number in $Met^{+}Hcy^{-}$ relative to $Met^{-}Hcy^{+}$ ($P < 0.001$; Table 2).

The MDI did not differ significantly among *BRCA1* and *BRCA2* carriers with or without breast cancer and noncarrier controls (Table 2); however, there was a trend for a lower MDI in carriers without breast cancer relative to the two other groups.

Polymorphisms in the *'de novo'* Methionine Synthesis Pathway. Table 3 shows the frequency of selected polymorphisms in methionine metabolism genes. The frequency distribution of wild-type homozygotes, heterozygotes, and variant allele homozygotes did not differ significantly among *BRCA1* and *BRCA2* mutation carriers with or without breast cancer and noncarrier controls for the *MTRR* A66G, *MTRR* C524T, *MTHFR* C677T, *MTHFR* A1298C, and *MTHFR* A1793G polymorphisms; however, for *MTR* A2756G, there was an increased frequency of G variant allele carriers in the BRCA mutation carriers with breast cancer relative to the noncarrier control group ($P = 0.009$) but no difference for other group comparisons.

Table 1. Pearson correlation of age and BMI with viable cell number and MDI on day 9

		Viable cell number in $Met^{+}Hcy^{-}$	Viable cell number in $Met^{-}Hcy^{+}$	MDI
Age	*r*	−0.520	−0.444	0.205
	P	< 0.001	< 0.001	0.102
BMI	*r*	−0.239	−0.164	0.228
	P	0.006	0.063	0.068

In *BRCA1* and *BRCA2* mutation carriers, the presence of the *MTR* A2756G mutation (i.e., AG or GG) and *MTHFR* C677T mutation (i.e., CT or TT) was associated with increased risk in breast cancer with an odds ratio of 3.2 ($P = 0.16$; 95% confidence interval, 0.76-13.9) and 3.9 ($P = 0.09$; 95% confidence interval, 0.93-16.3), respectively.

Table 4 displays the MDI of selected polymorphisms in methionine metabolism genes. *MTR, MTRR,* and *MTHFR* polymorphisms did not significantly affect MDI, except for *MTHFR* C677T, which was borderline significant ($P = 0.050$). When comparing *MTHFR* 677CC homozygotes with carriers of at least one T allele (i.e., CT or TT), MDI of T allele carriers was significantly increased (3.9 ± 0.4) relative to CC homozygotes (3.0 ± 0.2; $P = 0.017$).

Discussion

The study of MDP in familial cancer is important for two reasons: (*a*) MDP may reflect important differences in folate/methionine metabolism, which is a known risk factor for several cancers (29, 30), and (*b*) knowledge of MDP in normal somatic and cancer cells is essential to evaluate the feasibility of using methionine restriction as a strategy to selectively restrict cancer growth.

To the best of our knowledge, this is the first time that MDP has been studied in peripheral blood lymphocytes of *BRCA1* and *BRCA2* carriers with or without breast cancer. The relevance of determining whether MDP is a risk factor for breast cancer relates to the pressing need to identify who of the *BRCA1* and *BRCA2* carriers is at highest risk as this has implications on critical personal decisions relating to radical preventive surgery, i.e. mastectomy. Excessive proliferation of cells and altered methylation status is a common risk factor for a great variety of cancers and it is therefore plausible that a single potential risk factor that can affect both of these processes (such as MDP) might be relevant to the risk and etiology of both *BRCA1* and *BRCA2* breast cancers and, thus, justifies combining *BRCA1* and *BRCA2* carriers in the analysis.

Viable cell number in $Met^{+}Hcy^{-}$ and $Met^{-}Hcy^{+}$ medium as well as MDI did not differ significantly among carriers with or without breast cancer and

Table 2. Viable cell number ($\times 10^6$ cells/mL) in Met^+Hcy^- and Met^-Hcy^+ cultures and MDI of *BRCA1* and *BRCA2* mutation carriers with or without breast cancer and noncarrier controls, measured on day 9

	Non-carrier controls	Mutation carriers without breast cancer	Mutation carriers with breast cancer	ANCOVA P*
N	24	20	22	
Met^+Hcy^-	1.14 ± 0.03	1.25 ± 0.06	1.17 ± 0.05	0.511
Met^-Hcy^+	0.41 ± 0.03	0.52 ± 0.04	0.39 ± 0.03	0.227
t test P†	< 0.001	< 0. 001	< 0.001	
MDI ‡	3.43 ± 0.38	2.90 ± 0.26	3.44 ± 0.26	0.399

NOTE: Data displayed represent mean ± SE and have not been adjusted for age and BMI.
*Age and BMI were included as covariates in the ANCOVA analysis.
†The t test P values for comparison of growth in Met^+Hcy^- and Met^+Hcy^- was done to highlight the differences under these culture conditions.
‡The ratio of viable cell number in Met^+Hcy^- cultures relative to viable cell number in Met^-Hcy^+ cultures on day 9 provides the MDI measure as it reflects the relative degree to which cells have difficulty in dividing when methionine is replaced with its precursor homocysteine.

controls, suggesting no effect of MDP on breast cancer risk in *BRCA1* and *BRCA2* germline mutation carriers. Of the three comparisons, that between *BRCA1* and *BRCA2*, carriers with and without cancer is the most important because it addresses the key question of who is most at risk among those with a predisposition to breast cancer. The comparison with non-carriers without breast cancer may be informative if carriers without breast cancer show a unique characteristic that differs from the general population. In this regard, carriers without breast cancer tended to have the lowest MDI, which supports the hypothesis that MDI may be associated with their resistance to the disease. The trend for a 16% lower MDI in carriers without breast cancer relative to non-carrier controls and carriers with breast cancer provides a hint that lower MDI might be associated with reduced cancer risk in BRCA carriers, but the study was not sufficiently powered to verify this. Based on the observed SD of the data obtained, the study was adequately powered to detect an effect size difference of 40% at 80% power $P < 0.05$ for the MDI.

Furthermore, these results need to be interpreted with caution because methionine dependency is a feature that is not solely dependent on homocysteine metabolism but can also be due to limited supply of the other precursors of methionine, i.e. 4-methylthio-2-oxobutanoic acid and its precursor 5-methylthioadenosine (31). 5-Methylthioadenosine is formed when dcSAM transfers its aminopropyl moiety to putrescine and spermidine to synthesize spermidine and spermine, respectively. SAM carboxylase activity, which converts SAM into dcSAM, is increased in methionine-dependent cells when methionine is restricted to maintain polyamine levels (32, 33). Cells unable to synthesise methionine from 5-methylthioadenosine or homocysteine would be expected to exhibit reduced cell growth when methionine is restricted; however, because methionine dependency in the salvage pathway was not specifically assessed in the current study, it cannot be excluded that cells in this study, exhibiting MDP were not able to salvage methionine from 5-methylthioadenosine. In future studies, it will be necessary to assess growth not only in

Table 3. Observed distribution of BRCA mutation carriers with or without breast cancer and controls among genotypes for selected polymorphisms in methionine metabolism genes

		Non-carrier controls	Carriers without breast cancer	Carriers with breast cancer	χ^2 P
MTR A2756G	AA	18 (95)	13 (76)	9 (50)	0.259*
	AG	1 (5)	3 (18)	8 (44)	0.009 †
	GG	0 (0)	1 (6)	1 (6)	0.226 ‡
MTRR A66G	AA	1 (5)	4 (21)	3 (15)	0.181*
	AG	1 (5)	2 (11)	5 (25)	0.061 †
	GG	20 (90)	13 (68)	12 (60)	0.486 ‡
MTRR C524T	CC	7 (36)	7 (36)	5 (28)	0.920*
	CT	6 (32)	5 (28)	5 (28)	0.711 †
	TT	6 (32)	7 (36)	8 (44)	0.830 ‡
MTHFR C677T	CC	15 (68)	14 (77)	9 (47)	0.485*
	CT	3 (14)	3 (17)	6 (31)	0.318 †
	TT	4 (18)	1 (6)	4 (32)	0.145 ‡
MTHFR A1298C§	AA	10 (45)	5 (26)	4 (19)	0.330*,‖
	AC	12 (55)	14 (74)	17 (81)	0.104 †,‖
					0.712 ‡,‖
MTHFR A1793G§	AA	16 (72)	17 (89)	17 (89)	0.249*,‖
	AG	6 (28)	2 (11)	2 (11)	0.249 †,‖
					1.000 ‡,‖

NOTE: Numbers in parenthesis refer to % distribution.
*Comparison between non-carrier controls and carriers without breast cancer.
†Comparison between non-carrier controls and carriers with breast cancer.
‡Comparison between carriers with breast cancer and carriers without breast cancer.
§No homozygotes for the rare alleles in these genes were detected in this cohort.
‖Fisher's Exact P.

Table 4. MDI of selected polymorphisms in methionine metabolism genes

	AA*		Aa*		aa*		ANOVA *P**
MTR A2756G	(40)	3.36 ± 0.25	(12)	3.21 ± 0.41	(2)	3.56 ± 1.33	0.937
MTRR A66G	(8)	3.50 ± 0.53	(8)	3.92 ± 0.86	(44)	3.10 ± 0.19	0.324
MTRR C524T	(19)	3.07 ± 0.30	(16)	3.43 ± 0.52	(21)	3.36 ± 0.27	0.761
MTHFR C677T	(38)	2.95 ± 0.18	(12)	3.74 ± 0.62	(9)	4.12 ± 0.49	0.050
MTHFR A1298C*	(19)	3.61 ± 0.46	(42)	3.17 ± 0.19		–	0.295
MTHFR A1793G*	(50)	3.31 ± 0.22	(10)	3.06 ± 0.35		–	0.634

NOTE: Data displayed represent (*n*) and mean ± SE. *n* = number of subjects. #, AA, homozygotes for wild-type allele; Aa, heterozygotes; aa, homozygotes for rare variant allele.
*No homozygotes for the rare alleles in these genes were detected in this cohort.

$Met^{-}Hcy^{+}$ medium but also in medium deficient in methionine to which 5-methylthioadenosine is added to test the functionality of both the *de novo* and salvage pathway.

We cannot totally exclude that exposure to cancer chemotherapeutic agents might have influenced MDI measurements; however, none of the chemotherapeutic agents in breast cancer chemotherapy target the methionine, homocysteine, or 5-methyltetrahydrofolate synthesis reactions, which may influence the MDP measured in this study. In addition, because measurement of the MDI uses one's own cells as controls, any residual effects of chemotherapy on proliferation would have been equivalent in the $Met^{+}Hcy^{-}$ and the $Met^{-}Hcy^{+}$ cultures and therefore nullified when the MDI is calculated as a growth ratio. Furthermore, the MDI in the non-carrier controls, and the carriers with breast cancer were almost identical, which argues against an effect of chemotherapy.

Our results show that the ability of cells of *MTHFR* C677T mutant allele carriers to grow in absence of methionine is restricted as indicated by an increased MDI. This finding is consistent with the previously identified reduced MTHFR enzyme activity of the MTHFR C677T variant genotype (19, 24), which results in a reduction of the availability of 5-methyltetrahydrofolate as a methyl donor for the synthesis of methionine from homocysteine. A previous study by our group (8) did not show an association of *MTHFR* C677T and A1298C or *MTR* A2756G polymorphisms on nuclear division of peripheral blood lymphocytes in $Met^{-}Hcy^{+}$ in lymphocytes from healthy individuals. The absence of an effect of *MTHFR* C677T in the latter study was probably due to a less rigorous assessment of methionine dependence based on measuring proportion of cells undergoing nuclear division during a 48-h period rather than the actual accumulated viable cell number over a 9-day culture period used in our study. Further research into the influence of the *MTHFR* C677T polymorphism on methionine dependency is necessary as our study population was small and the concentration of riboflavin, the cofactor for MTHFR, in RPMI 1640 was supraphysiologic and may have minimized the difference in MTHFR activity due to the C677T polymorphism (34). Future studies with medium containing physiologic concentration of cofactors and/or substrates required in methionine metabolism such as 5-methyltetrahydrofolate, riboflavin, choline, and vitamin B_{12} are recommended to better reflect the *in vivo* situation.

In our study, *BRCA1* and *BRCA2* germline mutation carriers with breast cancer exhibited an increased number of *MTR* A2756G variant allele carriers relative to those without breast cancer and noncarrier controls. To date, several studies have investigated the possible link between *MTR* A2756G and breast cancer. One of these studies reported a reduced breast cancer risk in heterozygotes and homozygous variant allele carriers relative to common homozygotes (35), whereas the others did not find a significant association between *MTR* A2756G genotype and breast cancer (36-39). Therefore, our findings of an association of breast cancer with the *MTR* A2756G polymorphism may be relevant only to *BRCA1* and *BRCA2* families examined in our study. The plausibility of an association of the MTR A2756G polymorphism with breast cancer risk is supported by the studies of (*a*) Li et al. (40) who reported a strikingly reduced estrogen hormone receptor concentration in breast tumors homozygous for the *MTR* 2756G allele and (*b*) studies that show *BRCA1* and *BRCA2* breast tumors are characterized by a low estrogen receptor concentration (41, 42). This suggests that further studies into the association of *MTR* A2756G with breast cancer estrogen receptor phenotype, especially in *BRCA1* and *BRCA2* germline mutation carriers, may be required to verify a possible causal effect of MTR A2756G polymorphism on estrogen receptor expression.

The apparent association of the *MTHFR* C677T mutation with breast cancer risk observed in our study is consistent with the observations in large cohort studies in Japan (43), USA (37), Austria (44), Italy (45), Poland (46), China (47), and Australia (48); however, given the small group size and uncertainty due to multiple comparisons, the results from our study of an association of breast cancer risk with *MTR* A2756G and *MTHFR* C677T polymorphism can only be considered preliminary.

Taken together, the results of this study suggest a weak trend for increased breast cancer risk in *BRCA1* and *BRCA2* mutation carriers who exhibit increase methionine dependence and who also carry a *MTHFR* C677T and/or *MTR* A2756G variant allele.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Section 1734 solely to indicate this fact. pWe thank the participants who kindly donated their time and their blood samples to enable this research to be completed.

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Cancer Epidemiol Biomarkers Prev 2008;17:2565-2571.