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Abstract

Genes involved in DNA damage surveillance and repair are implicated in breast cancer susceptibility and in breast tumor pathology. We will test the hypothesis that the risk for more aggressive breast cancer is increased by nutritional deficiencies of folic acid/vitamin B12 and niacin. The study population will consist of 200 women (self-report as African-American or European-American) newly diagnosed with breast cancer in South Carolina. Dietary deficiencies of folic acid and niacin have been reported in populations of low socioeconomic status, such as reside in South Carolina. The specific objectives of the research are 1) to analyze the status of folic acid/vitamin B12 and niacin and the extent of DNA damage in peripheral blood lymphocytes from patients and 2) to analyze the association between these variables and clinical pathology of the resected tumor. A novel assay has been developed to analyze a key metabolite of folic acid, methylenetetrahydrofolate (MTHF), that is a determinant of genome stability. The levels of MTHF vary by 10-fold among patients with breast cancer. The associations among MTHF, the genes encoding the enzymes that partition MTHF, and tumor pathology is under investigation.

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INTRODUCTION

Histopathological grade, tumor size, and nodal status are well-documented prognostic factors in breast cancer (1). Breast cancer is associated with defects in DNA damage surveillance and repair. Polymorphism in genes encoding proteins involved in DNA repair has been reported to increase susceptibility to breast cancer (2). Genetic or environmental factors that increase the frequency or persistence of DNA damage are expected to increase the risk for breast carcinogenesis. Whether such factors are determinants of tumor pathology is unknown and is the focus of the proposed research. Deficiencies of niacin and folic acid/vitamin B12 are associated with DNA damage (3). In one investigation, 15-20% of women (n=687) on a Western diet exhibited niacin deficiency. Approximately 10% of the U.S. population have deficient folate intake, with significantly higher estimates in populations of low socioeconomic status (SES) (3). South Carolina, with median earned income of only 81% of the US average, has a long-standing history of both folate and niacin deficiency (4,5). Niacin deficiency results in a decrease in tissue levels of nicotinamide adenine dinucleotide (NAD), a substrate of poly(ADP-ribose) polymerase (PARP). Upon DNA damage, PARP catalyzes the ADP-ribosylation of proteins involved in DNA repair. In niacin deficiency, NAD is rapidly depleted and the response to DNA damage is abrogated. PARP-1 plays a regulatory role in the repair of strand breaks initiated by base excision repair (BER) (6). Deficiency of folic acid/vitamin B12 results in a decrease in tissue 5,10-methylenetetrahydrofolate (MTHF), which is partitioned between two pathways that play key roles in DNA integrity (Figure 1). In one, thymidylate synthase (TS) utilizes MTHF in the synthesis of thymidylate (TMP), an essential nucleotide in DNA. Depletion of TMP is associated with misincorporation of uracil in DNA and induction of BER-mediated strand breaks (7). In the second pathway, methylenetetrahydrofolate reductase (MTHFR) utilizes MTHF for the synthesis of methionine, which supplies the methyl group for DNA methylation. DNA hypomethylation is associated with a decrease in genome instability and is a frequent alteration in human cancers (8).

The research is focused on testing two novel hypotheses in a study population in South Carolina. First, the research will test the hypothesis that a more extensive DNA damage in lymphocytes is associated with higher incidence of breast tumors with poor prognoses. Second, the research will test the hypothesis that a deficiency of both niacin and folic acid/vitamin B12 is synergistic in the effect on DNA damage (i.e., a deficiency in TMP increases BER-mediated DNA damage and a deficiency in NAD reduces repair of this damage). The specific aims of the research are 1) to analyze the status of niacin and folic acid/vitamin B12 and the extent of DNA damage in circulating blood cells of patients with breast tumors and 2) to analyze the association between these variables and clinical pathology of the resected tumor. This pilot study has the potential to lead to intervention trials that directly address the role of nutritional status as a prognostic indicator in breast cancer and to facilitate the identification of additional factors (e.g., genetic) that modify disease outcome. Likewise, it can lead to the design of studies to validate the use of DNA damage in lymphocytes as a biomarker of disease prognosis. Because of the relatively high proportion of African-American women in our study population, the data can be extrapolated for future studies of the interrelationship between genetics, nutritional status, SES, and tumor pathology in women of differing ethnicity.

METHODS

Study Population: The study population consists of women newly diagnosed with breast cancer at the South Carolina Comprehensive Breast Center (SCCBC) at the Palmetto Health Alliance (PHA), Columbia, SC. This is a center that serves a region with a significant population of low SES. Patients are eligible for the study who are having surgical removal of their breast cancer at PHA (no systemic or radiation therapy prior to surgical biopsy), are female, 20-84 years of age, consider themselves either African-American (AA) or European-American, and have consented to donate their tissues for research purposes through a protocol for tissue/tumor banking previously approved by the PHA institutional review board. Peripheral blood, released by the tissue bank of the South Carolina Cancer Center (PHA Institutional Review Board #96-07, "A Cancer Tissue Bank for the South Carolina Cancer Center"), was obtained after approval by a regulatory committee. Blood was collected in tubes containing EDTA (4 ml) or heparin (8 ml). Packed red blood cells and plasma were isolated from EDTA tubes. Lymphocytes were isolated from heparinized tubes and cryopreserved in freezing medium (RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum and 10% DMSO). Blood fractions were frozen at -80°C .

Extraction of packed red blood cells: For extraction of 5,10-methylenetetrahydrofolate (MTHF), packed red blood cells were thawed in 50 mM Tris, pH 7.4- 1.0% ascorbate-0.2% Triton X-100. Suspensions were heated at 90°C for 10 min, then centrifuged at 13 K rpm at RT for 5 min. Supernatants were covered with argon and frozen at -70°C until analysis of MTHF. Extraction of $\text{NAD}^{+}/\text{NADP}^{+}$ was conducted as described previously (9). Briefly, frozen packed cells were solubilized in NaOH, which was rapidly neutralized with H_3PO_4 . Cell protein was precipitated with HClO_4 and extracts were neutralized with KOH and centrifuged to remove insoluble material before storage at -70°C .

Analysis of 5,10-Methylenetetrahydrofolate (MTHF): Recombinant thymidylate synthase (TS) from *Lactobacillus casei* was purified from the *Escherichia coli* strain $\chi 2913$ transformed with pKPTS-1 (10). The enzyme was purified by FPLC using Q-Sepharose as described previously (11) and stored at -20°C in a buffer containing 50 mM Tris, pH 7.4, 100 mM KCl, 4 mM DTT, and 15% glycerol. Enzyme activity was routinely analyzed as described previously (12). Red blood cell extracts were analyzed in a ligand binding assay containing 50 mM Tris-HCl, pH 7.4, 375 $\mu\text{g}/\text{ml}$ bovine serum albumin, 25 mM MgCl_2 , 0.15 μM $[6\text{-}^3\text{H}]\text{FdUMP}$ (15 Ci/mmol), and 0.1 μM *L. casei* TS. Unbound radioligand was removed by charcoal adsorption as described previously (12). MTHF was determined by using a standard curve (Figure 2), with a lower limit of detection of 20 fmoles. The data were normalized to the hematocrit determined from whole blood cell counts using a Sysmex K1000 analyzer.

DNA Damage Analysis: DNA damage in cryopreserved lymphocytes from patients was assessed by a single cell alkaline gel electrophoresis assay using the CometAssay reagent kit (Trevigen) (13). Cryopreserved lymphocytes were recovered by rapid thawing at 37°C . Cells (approximately 1000) were lysed, electrophoresed in alkaline gels for 25 min at 17 V (0.6 V/cm), and stained with SYBR Green according to the manufacturer's protocol. DNA was analyzed by fluorescence microscopy (Zeiss, Germany) equipped with filter sets appropriate for SYBR Green (excitation, 494; emission, 521). Images were captured and tail moments were analyzed using public domain software (14). Comets were classified into three categories (nominal, medium, or high intensity tail

DNA content. At least 75 cells were counted per slide. As an internal control, the human lymphoblastoid cell line, GM00131 (Coriell) was utilized. Only 2% of cells in the population exhibit random loss of 1 chromosome. Cells were cryopreserved using lymphocyte freezing medium and recovered and analyzed as described above.

Genotype Analysis: Genomic DNA was isolated from either whole blood or cryopreserved lymphocytes using a Promega DNA Isolation kit. A repeat length polymorphism in the promoter region of *TYMS* was analyzed as described previously with some modification (15). Briefly, genomic DNA (100ng) was incubated with forward primer, 5'-CGAGCAGGAAGAGGCGGAGC and reverse primer, 5'-GGCGGGGGGCAAGGGC, PCR products were separated by electrophoresis on 3% agarose gels, stained with ethidium bromide, and visualized by UV light. A SNP at position 677 in the methylenetetrahydrofolate reductase (*MTHFR*) gene was analyzed by a modified procedure (16) using the forward primer, 5'-AGGACGGTGCGGTGAGAGTG and reverse primer, 5'-ACCTGAAGCACTTGAAGGAGAAGGT. PCR products were digested with *Hinf*I and restriction fragments separated on 2% agarose gels, and visualized as described above. Digestion of the C677 allele yields a 210 nt fragment; digestion of the T677 allele yields fragments of 175 and 35 nt.

Data Analysis: A population size estimate of 200 women is based on an assumption of $\alpha=0.05$ with a 2-sided hypothesis test and an exposure prevalence of 20% [80% power to detect odds ratio (OR) =2.0; 63% power, OR=1.7; 47% power, OR=1.5]. Estimates of effect size and variability obtained will be used to refine power and sample size calculations in future studies. Clinicopathological data were obtained from the SC Central Cancer Registry (SCCCR) by linkage via databases managed by Clinical Trials (PHA) and the tissue bank at the South Carolina Cancer Center. All data were removed of identifiers before being returned to the investigative team prior to statistical analyses. Correlation and regression analyses will be performed using the SPSS statistical package.

RESULTS

The objective of the grant was to investigate the associations between nutritional status and DNA damage in lymphocytes and between DNA damage in lymphocytes and the clinicopathology of breast tumors from newly diagnosed patients in the midlands region of South Carolina. A major clinical resource for the study was blood samples from consenting patients to be obtained by staff of the clinical trials department and stored in the tumor bank at the South Carolina Cancer Center affiliated with the University. During the period between November, 2004 and July, 2005, the tissue bank and clinical trials department experienced changes in leadership and loss of key personnel and blood samples from patients newly diagnosed with breast cancer were not collected. The only blood samples available were from patients with breast cancer that had been treated with chemotherapy or radiation. Because the patients were exposed to therapy that could damage DNA, we were precluded from examining the central hypothesis of the proposal. Beginning in May, 2005, we received samples of whole blood, lymphocytes, or packed red blood cells from approximately 40 patients. Using samples from cases and blood donated by controls, we developed the tools that are necessary to conduct the proposed studies, including a novel assay to analyze MTHF, a key indicator of folate status in red blood cells. We also developed the assays for analyzing DNA damage in peripheral blood

lymphocytes. In late July, we were informed by the tumor bank manager that blood samples from newly diagnosed patients would be collected (10-20 samples/week). Two professional students in the College of Pharmacy have agreed to work with our lab to analyze the patient samples during the Fall and Spring semesters. Approximately 600 women are diagnosed with breast cancer at the SCCBC annually. Thus, we should have no problem obtaining sufficient studies to lend power to our analysis. We have submitted a request for a no-cost extension to support the students for this period and for expendable supplies. The manager of the tissue bank and the Director for Research and Education at the South Carolina Cancer Center are aware of the issues that delayed our studies and are committed to facilitating translational research. If requested, I will provide supporting letters from these administrators.

In our studies, we have developed a highly sensitive assay for analysis of MTHF in red blood cells. Because of the lability of MTHF, we conducted a number of control studies with donor blood to optimize the extraction of MTHF from red blood cells and to maintain the stability of MTHF in blood cell extracts. We observed that the range of MTHF in the sample population is approximately 10-fold. As described in the Introduction, MTHF is a critical metabolite involved in one-carbon transfers and is partitioned between two pathways that play an important role in genome stability. To enhance the likelihood that the data we are accumulating are published in a timely manner, we analyzed the genotype of *TYMS* and *MTHFR*, genes encoding the enzymes that partition MTHF through one or the other pathway. Both genes are polymorphic in humans and the alleles of these genes are associated with differences in either mRNA expression (TS) or enzyme activity (MTHFR), as well as differences in risk for colon and breast cancer (16-18). Interestingly, the *C677T* polymorphism in *MTHFR*, associated with decreased enzyme activity, is associated with an increase in risk for breast cancer and a decrease in risk for colon cancer. To date, no study of the association between alleles of these genes and clinicopathological features of breast tumors has been reported. More importantly, no analysis of an association between the alleles of these genes and MTHF levels has been reported. We conducted genotype analysis in DNA isolated from either whole blood or lymphocytes from the sample population. Although not proposed in the original application, the application submitted to the Institutional Review Board at PHA included an objective to isolate DNA from whole blood or lymphocytes for analysis of genotypes of genes that are associated with nutritional status. The data that we have collected is shown in Table 1 and associations among MTHF levels, the *TYMS* and *MTHFR* genotypes, and clinicopathological characteristics of the sample population are under analysis by Dr. Adams and Dr. Hebert.

KEY RESEARCH ACCOMPLISHMENTS

- developed a protocol to extract the labile folate, methylenetetrahydrofolate (MTHF) from red blood cells
- developed an assay to analyze MTHF with a lower limit of detection of 20 fmoles
- developed the procedures required to analyze DNA damage in lymphocytes and $\text{NAD}^+/\text{NADP}^+$ in red blood cells
- analyzed the genotype of *TYMS* and *MTHFR* that encode enzymes that partition MTHF into distinct pathways that contribute to genome stability

REPORTABLE OUTCOMES

- manuscript in preparation entitled "Associations among 5,10-methylenetetrahydrofolate, genotypes of *TYMS* and *MTHFR*, and Breast Tumor Pathology"
- research conducted by Mei Li was in partial fulfillment of the requirements for a M.S. degree in Basic Biomedical Sciences
- procedures developed in the laboratory have been standardized to foster the training of Pharm.D. students in the College of Pharmacy in clinical research

CONCLUSIONS

A sensitive assay has been developed to analyze a key indicator of folate status that is partitioned between two pathways that play a critical role in genome stability. The genotypes of *TYMS* and *MTHFR* that encode the enzymes at the branch points in these pathways have been determined and associations between these variables and tumor pathology are in progress. The procedures for analyzing DNA damage in peripheral blood lymphocytes and for determining niacin status in red blood cells have been developed for future studies of the interrelationships between nutritional status, DNA damage, and tumor pathology.

REFERENCES

1. Isaacs, C., Stearns, V., and Hayes, D.F. New prognostic factors for breast cancer recurrence. *Semin. Oncol.* **28**:53-67 (2001).
2. Goode, E.L., Ulrich, C.M., and Potter, J.D. Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **11**:1513-1530 (2002).
3. Ames, B.N. and Wakimoto, P. Are vitamin and mineral deficiencies a major cancer risk? *Nature Rev. Cancer* **2**:694-704 (2002).
4. Thompson, S.J., Torres, M.E., Stevenson, R.E., Dean, J.H., and Best, R.G. Periconceptional multivitamin folic acid use, dietary folate, total folate and risk of neural tube defects in South Carolina. *Ann. Epidemiol.* **13**:412-418 (2003).
5. Hawn, L.J., Guldán, G.J., Chillag, S., and Klein, L. A case of pellagra and a South Carolina history of the disorder. *J. S. C. Med. Assoc.* **99**:220-223 (2003).
6. Schultz, N., Lopez, E., Saleh-Gohari, N., and Helleday, T. Poly(ADP-ribose) polymerase (PARP-1) has a controlling role in homologous recombination. *Nucl. Acids Res.* **31**:4959-4964 (2003).
7. Ladner, R. The role of dUTPase and uracil-DNA repair in cancer chemotherapy. *Curr. Prot. Pept. Sci.* **2**:361-370 (2001).
8. Hoffman, M.J. and Schulz, W.A. Causes and consequences of DNA hypomethylation in human cancer. *Biochem. Cell Biol.* **83**:296-321 (2005).
9. Jacobson, E.L. and Jacobson M.K. Tissue NAD as a Biochemical measure of niacin status in humans. *Meth. Enzymol.* **280**:221-231 (1997).
10. Pinter, K., Davisson, V.J., and Santi, D.V. Cloning, sequencing, and expression of the *Lactobacillus casei* thymidylate synthase gene. *DNA* **7**:235-241 (1988).
11. Steadman, D.J., Spencer, H.T., Dunlap, R.B., and Berger, S.H. Substitution at residue 214 of human thymidylate synthase alters nucleotide binding and isomerization of ligand-protein complexes. *Biochemistry* **38**:5582-5587 (1999).

12. Davis, S.T. and Berger, S.H. Modulation of 5-fluoro-2'-deoxyuridine response by folinic acid in human colonic tumor cell lines: the role of thymidylate synthase. *Mol. Pharmacol.* 35:422-427 (1989).
13. Duthie, S.J., Pirie, L., Jenkinson, A.M.E., and Narayanan, S. Cryopreserved versus freshly isolated lymphocytes in human biomonitoring: endogenous and induced DNA damage, antioxidant status, and repair capacity. *Mutagenesis* 17:211-214 (2002).
14. Helma, C. and Uhl, M. A public domain image-analysis program for the single-cell gel-electrophoresis (comet) assay. *Mutat. Res.*, 466:9-15 (2000).
15. Yousef, A.-M., Davis, R.A., Sticca, R.P., and Berger, S.H. Structural analysis of cDNA encoding thymidylate synthase in hepatic metastases of human colorectal tumors. *Int. J. Colorectal Dis.* 16:318-325 (2001).
16. Shrubsole, M.J., Gao, Y.-T., Cai, Q., Shu, X.O., Dai, Q., Hebert, J.R., Jin, F., and Zheng, W. *MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: results from the Shanghai breast cancer study. *Cancer Epidemiol. Biomarkers Prev.* 13:190-196 (2004).
17. Chen, J., Gammon, M.D., Chan, W., Palomeque, C., Wetmur, J.G., Kabat, G.C., Teitelbaum, S.L., Britton, J.A., Terry, M.B., Neuget, A.I., and Santella, R.M. One-carbon metabolism, *MTHFR* polymorphisms, and risk of breast cancer. *Cancer Res.* 65:1606-1614 (2005).
18. Sharp, L. and Little, J. Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review. *Am. J. Epidemiol.* 159:423-443 (2004).

APPENDIX

Central Role of 5,10-Methylenetetrahydrofolate

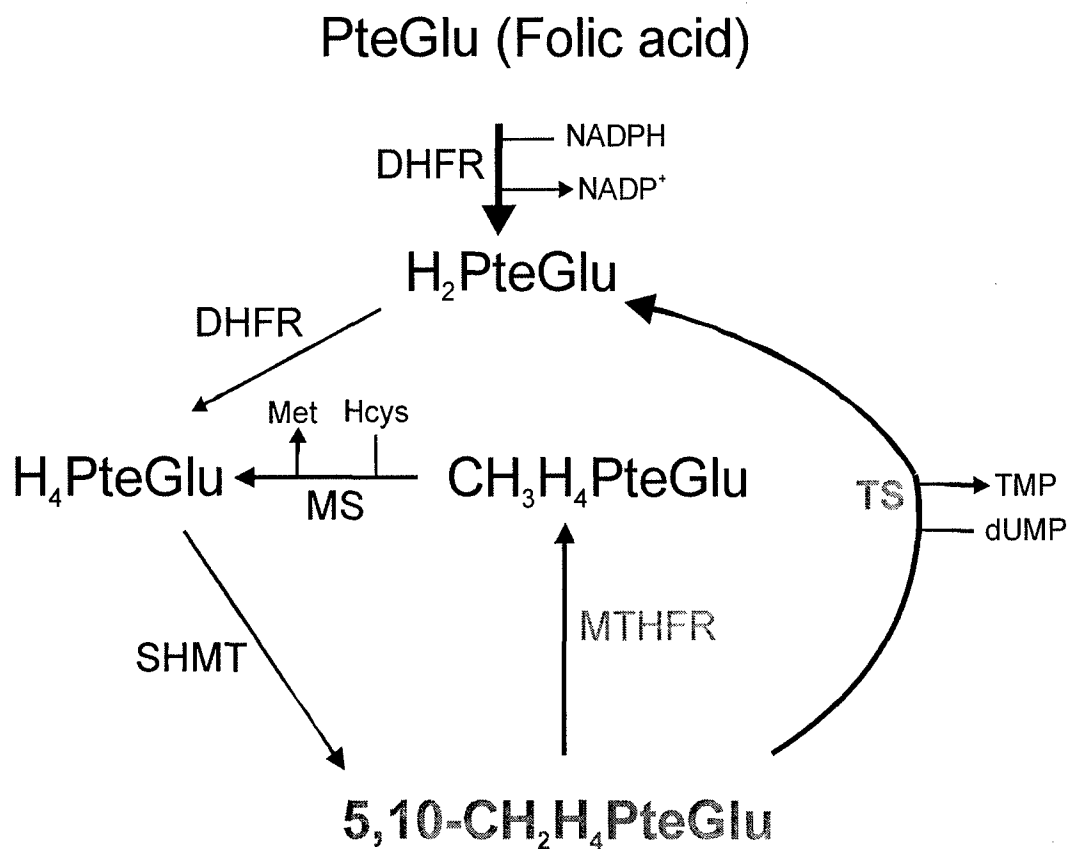


Figure 1

Standard Curve for Analysis of 5,10-Methylenetetrahydrofolate

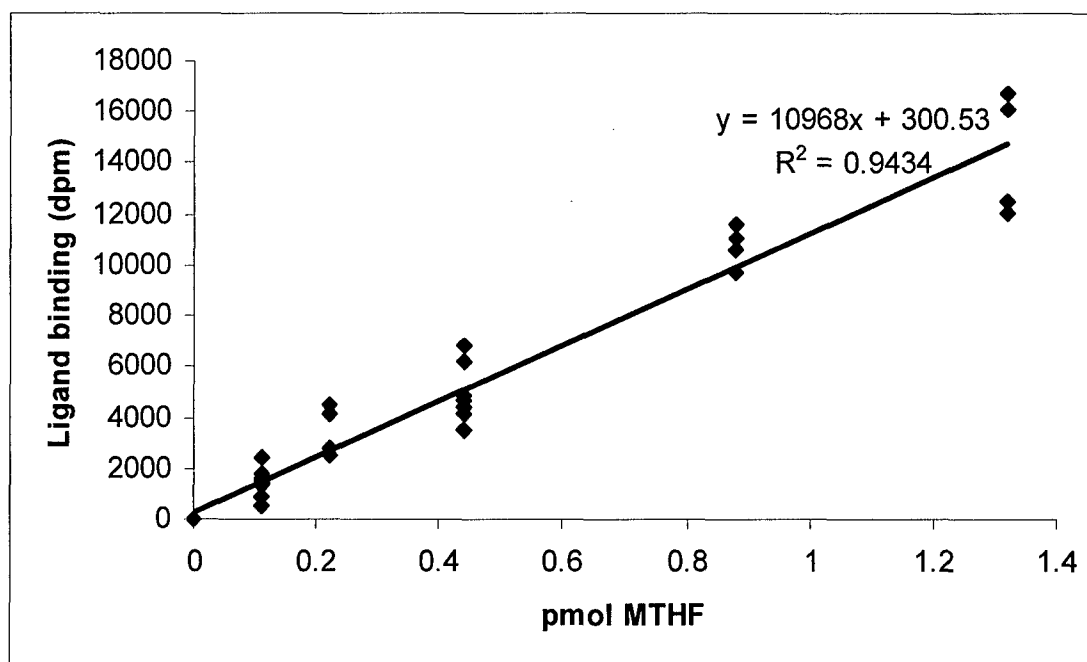


Figure 2

CH₂H₄PteGlu (MTHF) was determined by a ligand binding assay utilizing 0.15 μ M [6-³H]FdUMP (15 Ci/mmol) and 0.1 μ M *L. casei* TS. Unbound radioligand was removed by charcoal adsorption. The data are the mean of 4-6 separate experiments, each conducted in duplicate.

MTHF Levels, *TYMS* and *MTHFR* Genotype, and Clinicopathological Characteristics

ID	Ethnicity	Path Grade	ER status	<i>TYMS</i> Genotype	Node Status	AJCC Stage	<i>MTHFR</i> genotype	MTHF±SE (pmol/HCT)
1974	EA	I	positive	3R	Pos	IIB	C/C	
2115	EA	H	positive		Pos	IIIA	C/C	
4901	EA	I	positive	2R	Neg	I	C/C	0.269±0.021
4938	AA	I	positive	2R/3R	Neg	I	C/C	0.338±0.013
5215	AA	I	positive	2R/3R	Neg	IIA	C/C	0.453±0.017
5366	EA	I	positive	2R/3R	Neg	I	C/C	1.031±0.045
5528	AA	H	positive	2R/3R	Neg	IIA	C/C	1.711±0.065
7946	EA	H	negative	2R	Neg	I	C/C	0.549±0.027
8001	EA	L	positive	2R/3R	Neg	I	C/C	1.346±0.048
2733	EA	L	positive	2R	Pos	IIIA	C/T	0.169±0.007
2855	EA	H	negative	3R	Neg	IIA	C/T	1.76±0.003
2924	EA	I	positive	2R/3R	Neg	IIA	C/T	0.717±0.091
3550	EA	I	positive	3R	Pos	IIIA	C/T	0.43±0.069
4264	EA	H	negative	3R	Neg	I	C/T	
4268	EA	L	positive	3R	Pos	IIA	C/T	1.267±0.032
4888	EA	H	ND	2R	Neg	0	C/T	0.172±0.039
5097	EA	H	negative	2R/3R	Pos	IIB	C/T	0.823±0.059
5289	EA	H	negative	2R	Neg	IIA	C/T	
6667	EA	L	positive		Neg	I	C/T	0.492±0.011

7910	EA	I	negative	2R/3R	Neg	I	C/T	0.4±0.021
8002	EA	H	negative	3R	Neg	0	C/T	0.177±0.076
8734	EA	L	positive		Pos	IIA	C/T	1.261±0.08
8741	EA	H	negative	2R/3R	Pos	IIIA	C/T	1.078±0.044
9331	EA	H	negative	2R	Neg	IIA	C/T	
5208	EA	H	negative	2R	Neg	IIA	T/T	
7364	EA	L	positive	2R	Neg	I	T/T	1.682±0.032
2738	EA	I	positive	2R/3R	Neg	IIA		0.534±0.023
3021	EA	H	negative	3R	Neg	IIA		0.476±0.07
4258	EA	I	positive		Neg	I		0.515±0.002
4900	EA	I	positive	2R/3R	Neg	I		0.916±0.027
9240	AA	H	negative	2R/3R	Pos	IIIC		0.362±0.019
3677	AA	I	positive	2R/3R	Pos	IIIB		1.353±0.082
4273	EA	L	positive	2R/3R	Neg	I		1.345±0.082

Table 1

MTHF levels are the mean of at least 3 separate experiments, each conducted in duplicate.