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PRINCIPAL INVESTIGATOR: Sondra H. Berger, Ph.D.
Swann Adams, Ph.D.
James Hebert, Sc.D.
William Butler, M.D.
Mei Li

CONTRACTING ORGANIZATION: University of South Carolina
Columbia, SC 29208-0001

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14. ABSTRACT Genes involved in DNA damage surveillance and repair are implicated in breast cancer susceptibility and in breast tumor pathology. We are testing the hypothesis that the risk for more aggressive breast cancer is increased by nutritional deficiencies of folic acid and niacin. The study population consisted of 40 women (self-reported as African-American or European-American) previously diagnosed with breast cancer in South Carolina. The status of folic acid, measured as 5,10-methylenetetrahydrofolate (MTHF), and niacin, measured as nicotinamide adenine dinucleotide (NAD), was determined in circulating erythrocytes. Also analyzed were the genotypes of two genes encoding enzymes that partition MTHF into two pathways, which contribute to genomic integrity. A highly sensitive assay for detection of MTHF was developed; MTHF levels varied by 12-fold among the patients. An association was observed between genotype of methylenetetrahydrofolate reductase (MTHFR) and MTHF levels. This association was predicted by other investigations but not directly demonstrated. A novel association was observed between MTHFR genotype and ER status of the tumor. The data suggest that larger translational studies are warranted to validate the associations observed in this pilot 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 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 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 has been focused on testing two novel hypotheses in a study population in South Carolina. One hypothesis of interest is that more extensive DNA damage in peripheral lymphocytes is observed in women with a higher incidence of breast tumors with poor prognoses. The second hypothesis is that a deficiency of both niacin and folic acid 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 proposed research were 1) to analyze the status of niacin and folic acid 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 South Carolina, 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 consisted of women previously 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 were eligible for the study who had surgical removal of their breast cancer at PHA (no systemic or radiation therapy prior to surgical biopsy), are female and 20-84 years of age at time of diagnosis, self-reported as either African-American (AA) or European-American, and 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. Tubes containing EDTA (4 ml) or heparin (8 ml) were collected and blood 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 nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (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 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 $\mu\text{g/ml}$ 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.

Analysis of NAD/NADP: NAD and NADP in extracts from packed red blood cells were analyzed by enzymatic cycling assays as described previously (9). Briefly, NAD was measured in assays containing 0.11 M bicine, pH 7.8, 0.57 M ethanol, 0.48 mM methylthiazolyldiphenyl-tetrazolium bromide (MTT), 4.8 mM EDTA, 1 mg/ml bovine serum albumin (BSA), 50 $\mu\text{g/ml}$ (U) alcohol dehydrogenase (yeast; 507 U/mg prot), and 1.9 mM phenazine ethosulfate. For analysis of NADP, assays contained 76 mM phosphate buffer, pH 6.8, 7.1 mM isocitrate, 0.48 mM MTT, 7 mM MgCl_2 , 1 mg/ml BSA, 133 $\mu\text{g/ml}$ NADP-specific isocitrate dehydrogenase (porcine heart; 26-29 U/mg prot), and 1.9 mM phenazine ethosulfate. After incubation of assays for NAD or NADP at 30°C for 15 min, iodoacetate was added at a final concentration of 6 mM. The absorbance was determined at 570/600 nm using a microtiter plate reader. For standard

curves, concentrations ranging from 0 to 100 nM NAD or NADP were utilized. A representative standard curve for NADP is shown in Figure 3.

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 for minimal damage, 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. As a control for extensive damage, GM00131 cells were exposed *in vitro* to 200 μ M H₂O₂ at 4°C for 5 min.

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. The tumor characteristics of the sample population is shown in Table 1. 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.

PROBLEMS/ ALTERNATIVE STRATEGIES

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 October, 2005, we were only able to obtain samples of whole blood, lymphocytes, or packed red blood cells (RBCs) from 40 patients with breast cancer that had been previously treated with chemotherapy or radiation. Because the patients were exposed to therapy that could damage DNA, we were precluded from examining the two hypotheses of the proposal. Goals related to the statement of work that were accomplished include development and validation of methods for extraction of biochemicals [5,10-methylenetetrahydrofolate (MTHF), nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) from RBCs, development and validation of an assay to analyze MTHF, and analysis of MTHF levels in RBCs. Given the inability to address the key hypotheses with the patient samples, two strategies were taken. To generate new hypotheses, analyses of the genotypes of two polymorphic enzymes, thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR), that regulate the levels of MTHF, were conducted in lymphocytes from the patients. The IRB protocol for the study was modified accordingly. The second strategy was to collect blood samples from newly diagnosed patients. During the period between October, 2005 and April, 2006, samples from 49 patients were collected. During this period, we also analyzed NAD and NADP in the samples from the original 40 patients, developed the assays for analyzing DNA damage in peripheral blood lymphocytes, and conducted statistical analyses of the data collected on the original 40 patients. In April, 2006, the University suffered a major power outage and the biological samples in storage were allowed to thaw from -80°C to -45°C . Quality control studies revealed that NADP levels in packed red blood cells are unaffected but that extensive DNA damage has occurred in cryopreserved lymphocytes. After this devastating loss, our current strategies are two-fold: 1) to address the hypothesis that a deficiency of folic acid and niacin is synergistic in its effect on DNA damage by using a human cell line model; 2) to follow-up on new hypotheses generated by studies of the relationship between genotype and levels of MTHF.

RESULTS AND DISCUSSION

The levels of the folic acid derivative, 5,10-methylenetetrahydrofolate (MTHF) in RBCs have not been reported in human subjects. MTHF is labile and its concentration is low in tissues; however, it is the critical substrate in two pathways that play key roles in DNA integrity and genomic stability. A highly sensitive radioligand binding assay was developed to analyze MTHF (Figure 2). The range of MTHF in the sample population was approximately 12-fold, from 0.17-2.0 pmol normalized to the hematocrit (HCT). In the same population, the genotype of the genes encoding TS and MTHFR, the enzymes that partition MTHF through one or the other pathway was analyzed. The allele frequencies for the two loci are shown in Table 2. Genotype frequencies at both loci were similar to the frequencies reported for comparable populations (19-23). Analysis of allele frequencies by ethnicity revealed that the frequency of *CT* heterozygotes and of the variant *T* allele of *MTHFR* in European-American (EA) women is higher than in comparable populations, although the small population size makes the significance unclear (Figure 4). The *T* allele was observed to be significantly ($p < 0.5$) more common in tumors of low pathological grade, a result likely influenced by the higher frequency of *T* alleles in EA than AA women (35 and 14%, respectively). Significant associations were not observed between *T* allele frequency and nodal status; however, a higher frequency of the *T* allele was observed in patients with estrogen receptor-negative tumors. Of particular interest is the observation that MTHF levels are significantly higher ($p < 0.5$) in women homozygous for the *T* allele (Figure 5). Although an association between expression of the *T* allele and elevated levels of MTHF has been postulated and investigated by epidemiological investigations, this is the first direct evidence for such an association (24). Studies are warranted with a larger population size to validate this and other observations in this pilot study. Significant associations were not observed between the *TS* polymorphism and either MTHF levels or clinicopathological features.

The levels of NAD and NADP were analyzed in RBCs. The ratio of NAD/NADP (niacin number) varied 7-fold among the samples. Values ranged from 68.38- 466.42, with a mean niacin number of 238.3. Niacin numbers for a population of healthy adults ($n=687$) in Sweden in 1997 ranged from 28-337, with a mean of 160 (9). The niacin number is apparently comparable to that of normal adults. Using the standard deviation published in the investigation in Sweden, 14% of the patients in our population had niacin numbers below 1 standard deviation. Significant associations were not observed among niacin numbers and either MTHF levels or clinicopathological features of the tumors.

In summary, a significant association among MTHF levels, niacin number, and clinicopathological features was not observed. It is premature to conclude that MTHF and NAD levels are neither interdependent nor play a contributing role in breast tumor biology and behavior. The sample size in the pilot study is small and data were obtained from blood samples from patients several years after diagnosis. These are confounding factors. From our perspective, the extent of DNA damage in normal blood tissues is a link between nutritional status and tumor biology; thus, our inability to obtain data on damage in peripheral lymphocytes has been frustrating. We remain committed to examining the interdependency of MTHF and NAD in genome stability and are investigating this association in a human cell line model. We are also analyzing the association between MTHF levels in RBCs and *MTHFR* genotype in a larger sample population, as this association may underlie the reported effects of *MTHFR* genotype on risk for cancer and response to cancer therapy (24,25).

KEY RESEARCH ACCOMPLISHMENTS

- Coordinated sample acquisition and processing with tissue bank to streamline translational research in breast cancer
- Developed and validated methods for extraction of MTHF, NAD, and NADP from the same sample of RBCs
- Developed a sensitive assay for analysis of MTHF in RBC extracts
- Modified a published method for genotyping *MTHFR* at 677
- Analyzed the genotype frequencies of the 2R/3R repeat polymorphism in *TS* and C677T polymorphism in *MTHFR*
- Analyzed NAD and NADP in RBC extracts using techniques developed based on published methods
- Developed and validated the comet assay for measuring DNA damage in cryopreserved lymphocytes
- Analyzed associations among biochemical data, genotypes, and clinicopathological data

REPORTABLE OUTCOMES

- Data presentation: Li, M., Adams, S., Hebert, J., Zhang, J., Butler, W., and Berger, S. Nutritional Status and Tumor Pathology. Department of Defense Breast Cancer Research Program meeting, June 8-11, 2005, Philadelphia, PA
- Published abstract and data presentation: Luo, B., Li, M., Adams, S., Hebert, J., and Berger, S. Status of Two Key One-Carbon Transfer Pathways in Breast Tumors. Proc. Amer. Assoc. Cancer Res. 47:5392 (2006); data presented at 97th annual meeting of American Association for Cancer Research, Washington, D.C., April 1-5, 2006.
- Partial fulfillment of requirements for a M.S. degree, Mei Li; defense date, 09/29/06
- Partial fulfillment of requirement for a Ph.D. degree, BeiBei Luo; anticipated defense date, 12/09
- Training in laboratory techniques for two professional students in the Pharm.D. program of the USC College of Pharmacy, Vishal Shah and K. Sloan Hepfer; expected graduation date, 5/08
- Established a tissue and serum repository of newly diagnosed breast cancer patients; serum is being used by other investigators

CONCLUSION

The most important finding of the research is the association between homozygous expression of the variant *MTHFR* 677T allele and elevated MTHF levels. This association has been postulated and examined indirectly in multiple investigations; however, it has not been directly tested because of the inherent difficulty in analyzing MTHF levels in tissues. In this pilot study, strong associations were not observed among MTHF levels, niacin number, and clinicopathological characteristics of breast tumors from the patient set.

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APPENDIX

Central Role of 5,10-Methylenetetrahydrofolate

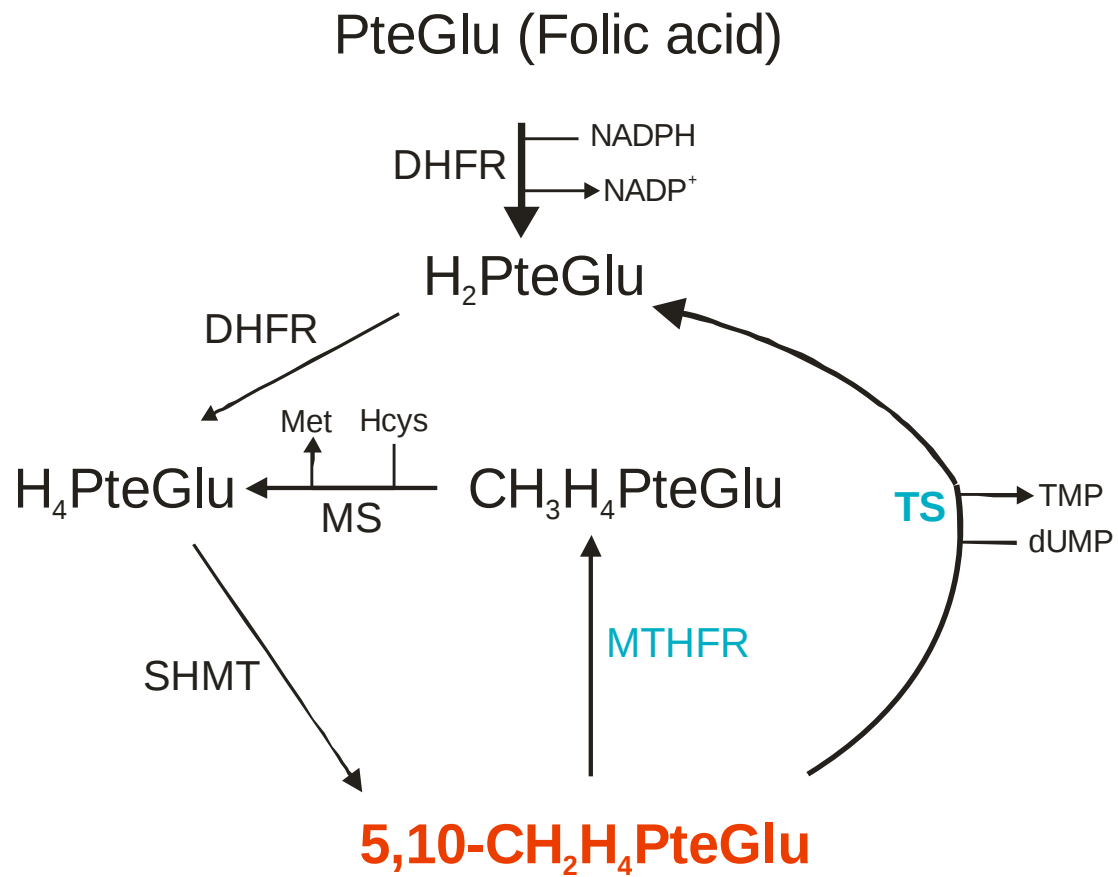


Figure 1

Standard Curve for Analysis of 5,10-Methylenetetrahydrofolate

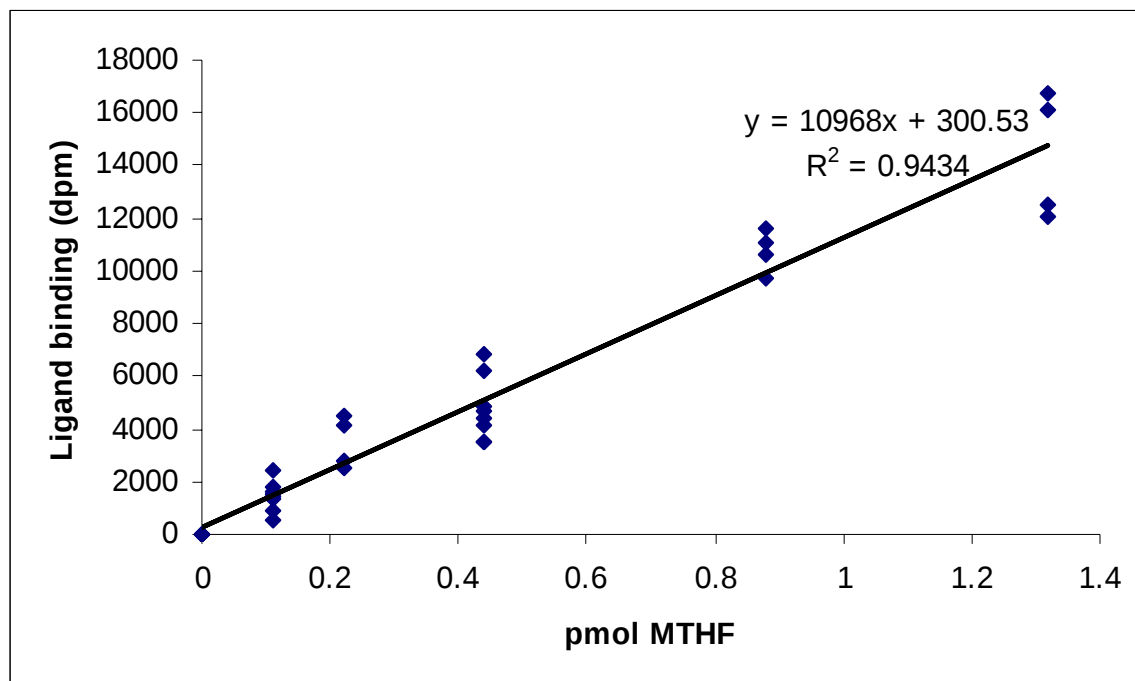


Figure 2

CH₂H₄PteGlu (MTHF) was determined by a ligand binding assay as described in Methods. The data are the mean of 4-6 separate experiments, each conducted in duplicate.

Standard Curve for Analysis of NADP

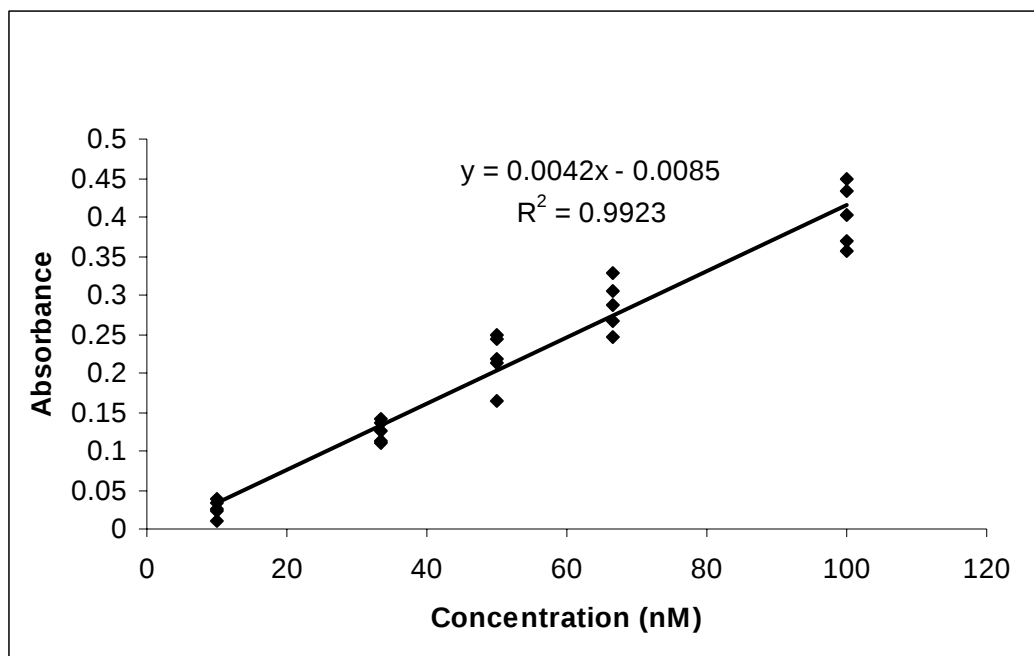


Figure 3

NADP was determined in an enzymatic cycling assay as described in Methods. The data are the result of 2 independent assays each conducted in quadruplicate.

Distribution of *MTHFR* C677T polymorphism

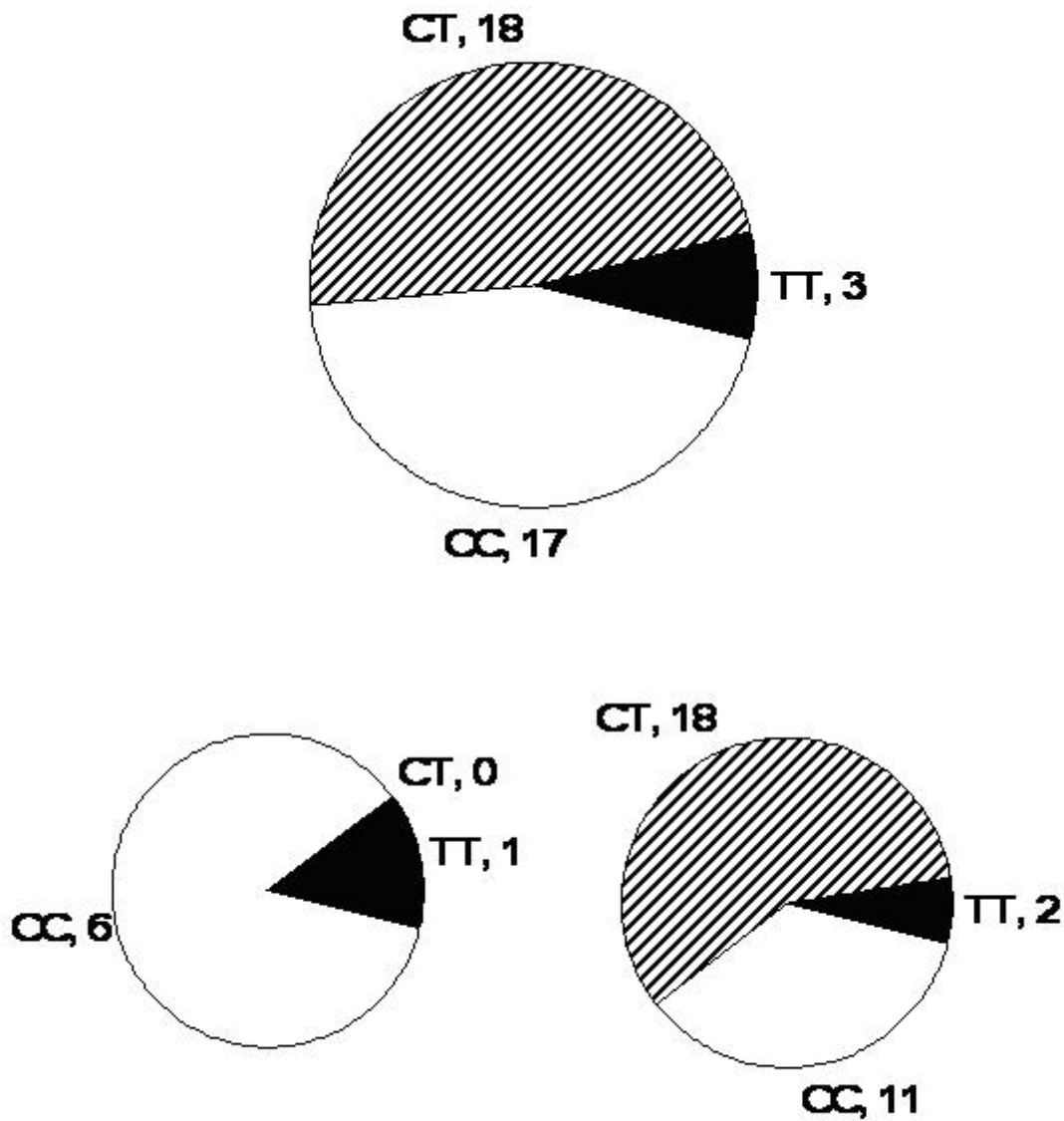


Figure 4

The distribution of *MTHFR* C677T polymorphism in the sample population (n=38; upper pie diagram) and stratified by ethnicity (African-American; bottom left) and (European-American; bottom right) is shown.

Association between MTHF Levels and *MTHFR* Genotype

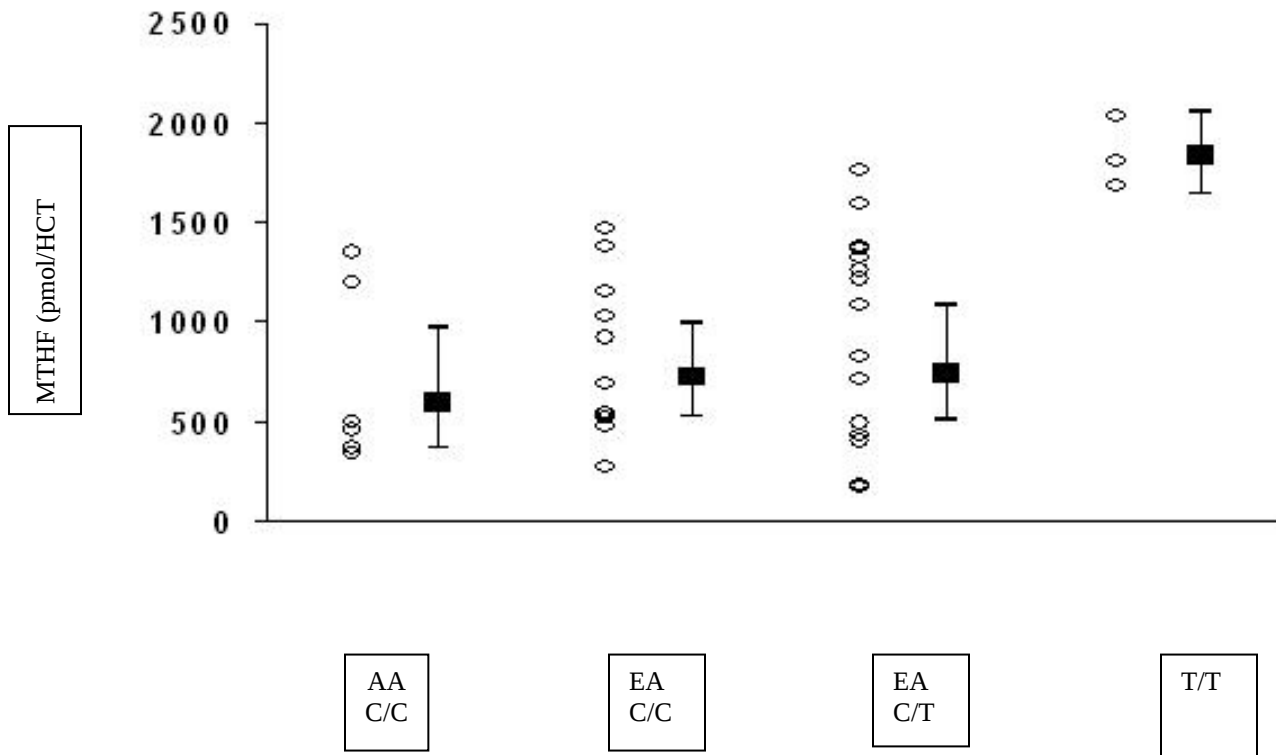


Figure 5

5,10-Methylenetetrahydrofolate (MTHF) levels in individual patients (designated by open circles) were segregated by *MTHFR* C677T genotype and ethnicity. Means are designated by solid squares and 95% CI by vertical bars. AA, African-American; EA, European-American

Table 1. Clinicopathological Characteristics of Breast Tumors

Histopathological Grade

I (^a LG)	11
II (IG)	13
III (HG)	16

AJCC Stage

0	2
I	14
II	15
III	9

Nodal Status

Negative	25
Positive	15

^bER Status

Positive	25
Negative	14
NA	1

^aLG, low grade; IG, intermediate grade; HG, high grade

^bER, estrogen receptor

NA not available

Table 2: Genotype Frequencies in Breast Cancer Patients

Tandem repeat polymorphism in *TS*

2R/2R	23%
2R/3R	54%
3R/3R	23%

677C/T polymorphism in *MTHFR*

CC	45%
CT	47%
TT	8%