A Phase II Trial of Pemetrexed in Advanced Breast Cancer: Clinical Response and Association with Molecular Target Expression


Abstract

Purpose: This phase II trial of pemetrexed explored potential correlations between treatment outcome (antitumor activity) and molecular target expression.

Experimental Design: Chemonaive patients with advanced breast cancer received up to three cycles of pemetrexed 500 mg/m² (10-minute i.v. infusion) on day 1 of a 21-day cycle, with folic acid and vitamin B₁₂ supplementation. Tumors were surgically removed after the last cycle of pemetrexed as clinically indicated. Biopsies were taken at baseline, 24 hours after infusion in cycle 1, and after cycle 3.

Results: Sixty-one women (median age, 46 years; range, 32-72 years) were treated and were evaluable for response. Objective response rate was 31%. Simple logistic regression suggested a potential relationship between mRNA expression of thymidylate synthase (TS) and pemetrexed response ($P = 0.103$). Based on threshold analysis, patients with “low” baseline TS ($<71$) were more likely to respond to pemetrexed than patients with “high” baseline TS ($>71$). Expression of baseline dihydrofolate reductase and glycinamide ribonucleotide formyl transferase tended to be higher in responders but this association was not significant ($P > 0.311$). TS expression increased significantly between baseline and biopsy 2 ($P = 0.004$) and dropped to near baseline levels at biopsy 3. Conversely, dihydrofolate reductase and glycinamide ribonucleotide formyl transferase decreased after pemetrexed chemotherapy.

Conclusions: Our results suggest a potential association between “low” pretreatment TS expression levels and response to pemetrexed chemotherapy. Future trials examining expression levels of other genes important to the folate pathway and/or breast cancer may identify a more robust multigene profile that can better predict response to this novel antifolate.

Primary chemotherapy has an expanding role in the treatment of unresectable locally advanced as well as advanced breast cancer. In addition to providing an opportunity to downstage advanced disease without compromising clinical outcome, primary chemotherapy has been useful in translational research studies for identifying molecular biomarkers that predict improved efficacy (1–3).

The hope of clinical translational research is to further improve on the empirical approach to cancer treatment. Historically, chemotherapy regimens have been designed from phase I studies that evaluate dose-limiting toxicities and potential antitumor activity. More recently, translational research studies have included analyses to identify molecular biomarkers that correlate with response and/or toxicity. The ultimate goal of this approach is to create individualized treatment regimens that offer an improved therapeutic profile by identifying patients most likely (or least likely) to benefit from a specific therapy.

Pemetrexed, a novel antifolate shown in vitro to target at least three different enzymes in the folate pathway [thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyl transferase (GARFT); refs. 4, 5], has shown clinical antitumor activity in a variety of tumor types (5). This agent was recently approved in the United States and the European Union for the treatment of advanced mesothelioma and non–small-cell lung cancer (6–8). Single-agent pemetrexed (with or without vitamin supplementation) was evaluated in previously treated breast cancer patients in four phase II trials (9–12). The overall response rates ranged from 10% to 28% and the toxicities were manageable, providing a rationale to initiate a clinical trial in chemotherapy-naive patients. A multivariate analysis conducted with data from early-phase pemetrexed trials established an association between an increased risk of severe pemetrexed-related toxicity and elevated serum homocysteine and/or elevated methylmalonic acid (13). Thus, since December 1999, folic acid and...
vitamin B₁₂ supplementation has been included in all pemetrexed-based regimens. In vitro studies indicated that tumor cell lines expressing high levels of TS or DHFR have reduced sensitivity to pemetrexed, suggesting that increased expression levels might correlate with reduced clinical efficacy (14, 15). In a clinical study using 5-fluorouracil (5-FU)/folinic acid for the treatment of gastric or colorectal cancer, patients with high TS-expressing tumors responded poorly to chemotherapy (16). Additional clinical studies evaluating 5-FU-based therapy in patients with metastatic colorectal cancer (17, 18), non-small-cell lung cancer (19), and breast cancer (20) indicated that lower pretreatment levels of TS protein were predictive of response to chemotherapy. Two large clinical studies in colorectal cancer confirmed the role of TS as a predictor of response to 5-FU and further showed that TS is a prognostic factor for survival and disease-free survival (independent of Dukes' stage) in patients treated with surgery alone (21, 22).

Drug-induced elevation of TS expression has been cited as a possible mechanism for developing resistance to chemotherapy (23). In vitro studies of human colon cancer cell lines showed induction of TS protein expression after short-term exposure (8-36 hours) to 5-FU (24). Notably, in that study, there was no associated change in TS mRNA expression.

In the present phase II clinical trial of single-agent pemetrexed (Alimta, Eli Lilly and Company, Indianapolis, IN) in chemonaive patients with unresectable locally advanced or metastatic breast cancer, we used molecular assays to better understand the relationship between baseline mRNA expression levels for TS, DHFR, and GARFT and response to chemotherapy. We also examined the potential modulation of target expression after pemetrexed treatment.

**Patients and Methods**

**Patient selection**

Chemotherapy-naive female patients, ≥18 years of age, with advanced (T₄ and N₀-N₂, M₀, M₁) breast cancer were included. Other inclusion criteria were Karnofsky performance status ≥70, adequate organ function (bone marrow reserve, hepatic, and renal), and a life expectancy of ≥12 weeks. Evidence of bidimensionally measurable lesions as determined by computed tomography, magnetic resonance imaging, or palpation was required. Pregnant women were not eligible and adequate contraception was required during the study and for 3 months after pemetrexed treatment. Patients were excluded if they were unable to interrupt aspirin or nonsteroidal anti-inflammatory drug therapy for 2 days before, the day of pemetrexed therapy, and 2 days after pemetrexed therapy and/or if they were unable to take folic acid and vitamin B₁₂ supplementation. Institutional ethics review boards approved the protocol and the trial was conducted according to the guidelines for good clinical practice and the Declaration of Helsinki. All patients provided written informed consent before treatment.

**Study design**

The primary goal of this single-arm, open-label, phase II study was to explore the relationship between response to pemetrexed and baseline mRNA expression of TS, DHFR, and GARFT. Secondary objectives included (a) determination of objective response rate after three cycles of pemetrexed; (b) characterization and quantification of drug-related toxicities; (c) evaluation of chemotherapy-associated changes in TS, DHFR, and GARFT mRNA expression during pemetrexed treatment; and (d) evaluation of a potential relationship between HER-2 gene amplification status and response to pemetrexed therapy.

Molecular assays were conducted with fresh tumor samples collected at three time points during the study. A pretreatment diagnostic tumor biopsy was taken ≥5 days after initiation of vitamin supplementation; a second biopsy was collected ~24 hours after the first pemetrexed dose; and a third biopsy was taken after the last dose of pemetrexed. After each biopsy, a small portion of tissue was embedded in frozen section support material and frozen in liquid nitrogen. Frozen tissue specimens were shipped to the Diagnostic Cytology Laboratory (Indianapolis, IN), where histology was confirmed and samples were later sliced for frozen storage (~70°C) or distribution for molecular biomarker analysis.

**Treatment plan**

Pemetrexed 500 mg/m² was given as a 10-minute i.v. infusion on day 1 of a 21-day cycle. Patients received up to three cycles of treatment. All patients were instructed to take oral daily doses of folinic acid (350-1,000 µg) beginning 5 to 7 days before the first pretreatment biopsy and then daily while on therapy. Vitamin B₁₂ (1,000 µg) was given by i.m. injection 24 hours after the first pretreatment biopsy and at 9-week intervals throughout pemetrexed treatment. Dexamethasone (4 mg orally, twice daily) was given the day before, the day of, and the day after each dose of pemetrexed therapy to prevent skin rash. Tumors were surgically removed following the last cycle of pemetrexed therapy when possible and clinically indicated.

Pemetrexed doses were delayed and modified if a patient experienced unacceptable hematologic toxicities (ANC <0.5 × 10⁹/L and platelets ≥50 × 10⁹/L required a 25% dose reduction; platelets <50 × 10⁹/L required a 50% dose reduction). Similarly, treatment was delayed when a patient experienced Common Toxicity Criteria grade 3/4 non-hematologic toxicities (except grade 3 transaminase elevations), including diarrhea. When nonhematologic symptoms resolved, therapy was resumed at 75% of the previous dose. Pemetrexed therapy was delayed for up to 42 days for patients with calculated creatinine clearance <45 mL/min. Patients requiring >42 days recovery time discontinued study therapy.

**Patient assessments**

Baseline tumor measurements (using computed tomography, breast magnetic resonance imaging, and palpation) were taken no more than 2 weeks before treatment. All patients had radiologic evaluations of lungs and liver. Tumor size (determined using computed tomography or magnetic resonance imaging) was evaluated every cycle. Assessments of objective response status and overall best tumor response were completed every 3 weeks throughout the study using Southwest Oncology Group standard response criteria (25). Briefly, complete response was defined as the disappearance of signs and symptoms of disease without the appearance of new lesions. Partial response was defined as a ≥50% reduction in the sum of the products of the perpendicular diameters of all measurable lesions without the appearance of new lesions or an increase in the size of existing lesions. Progressive disease was defined as either a 50% or 10-cm² increase in the sum of the products of all measurable lesions divided by the smallest sum observed, worsening of evaluable disease, reappearance of old lesions, or appearance of a new lesion. Patients who did not qualify as complete response, partial response, or progressive disease were considered to have stable disease. Patients who showed disease progression while on study were withdrawn from the study and immediately switched to a standard treatment. Independent review of response was not done.

Pretreatment biopsies were assayed by immunohistochemistry to determine hormone receptor status (estrogen receptor and progesterone receptor). Body surface area calculations and performance status evaluations were completed before each cycle. Clinical laboratory tests including hematology, blood chemistry, and creatinine clearance were completed before each cycle. Toxicities were graded using version 2.0 of the National Cancer Institute Common Toxicity Criteria scale during a follow-up safety evaluation ~30 days after the last therapy dose (26).
Molecular assays

Quantitative reverse transcription-PCR. Total RNA was extracted from fifteen 4-μm slices of frozen tissue taken from each biopsy sample. The tissue was lysed with 4 mol/L guanidine thiocyanate solution (27) and RNA was extracted using the Gentra PureScript RNA Purification kit (Gentra Systems, Inc., Minneapolis, MN). Contaminating DNA was removed before the reverse transcription reaction by treating samples with 2 units of DNase I (Ambion, Austin, TX; according to the protocol of the manufacturer). The different target mRNAs were quantified in individual reactions using the 5' nuclease assay [TaqMan sequence detection chemistry, Applied Biosystems (ABI), Foster City, CA]. The target-specific primer sequences used are shown in Table 1. The total volume was 20 μL for the reverse transcription reaction with final concentrations of random hexamers at 50 ng/μL and reverse target primer at 5 μmol/L. Reverse transcription reaction conditions included incubation for 10 minutes at room temperature with 30 units of ThermoScript enzyme (Invitrogen, Carlsbad, CA), followed by 50 minutes at 55°C and 5 minutes at 85°C. Subsequent PCR amplification for each target was conducted in a 25-μL total reaction volume containing 20 μL of PCR premix and 5 μL of cDNA template (from the reverse transcription reaction). The premix included TaqMan Universal PCR mix, target-specific forward and reverse primers (400 nmol/L for TS and GARFT and 200 nmol/L for DHFR), the appropriate TaqMan probes (200 nmol/L), and water. All TaqMan probes were labeled with fluorescent dye FAM on the 5' end and with the quencher dye TAMRA on the 3' end. Quantitative reverse transcription-PCR was conducted with the ABI Prism 7900 instrument using the following conditions: 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles alternating from 95°C for 15 seconds and 65°C for 1 minute. Target gene expression was normalized for the expression of β-actin, which was quantified using Pre-Developed Assay Reagents (ABI).

Fluorescence in situ hybridization. A fluorescence in situ hybridization assay was used to detect HER-2/neu gene amplification in formalin-fixed, paraffin-embedded tissues (PathVysion HER-2 DNA Probe Kit, Vysis, Abbott Laboratories, Downers Grove, IL). The locus-specific (17q11.2-q12) identifier probe was LSI HER-2/neu SpectrumOrange and the Chromosome Enumeration Probe [specific for the α satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1)] was CEP 17 SpectrumGreen. Tumor specimens were sliced and placed on slides at Diagnostic Cytology Laboratory and the PathVysion fluorescence in situ hybridization assay was conducted by researchers at Rush Presbyterian St. Luke’s Medical Center (Chicago, IL).

The original study plan also included immunohistochemical analysis of p53 protein with the rationale that p53 protein accumulation would reflect the mutation status in these breast biopsy samples. However, an updated review of these methods, published after the study began, revealed that newly designed DNA-based assays using techniques such as denaturing high-performance liquid chromatography yielded more accurate and specific information about mutation status (28). These techniques have been applied to our specimens and analyses of these data are ongoing.

Statistical considerations

Enrolled patients who received at least one dose of pemetrexed were evaluable for safety and efficacy. Because this was the first clinical study to evaluate potential correlations between baseline biomarker expression and response to pemetrexed chemotherapy, statistical analyses of biomarker data were done primarily for the purpose of hypothesis generation and to estimate parameters for use in future pharmacogenomic trials. The initial sample size and power analysis was based on a multiple logistic regression model (29) that included three molecular markers linked to pemetrexed activity (TS, DHFR, and GARFT with TS as the “flagship” marker). Retrospective analysis, however, revealed that the original model significantly underestimated the variability of TS expression (thereby overestimating the size of the treatment effect). A revised power calculation, which assumed that half of the patients had elevated TS expression (with a 5% response rate) and the other half had “low” TS expression (with a 25% response rate), showed that the given sample size enabled only a 35% probability of detecting a significant difference in response rates between TS groups (with z = 0.05).

Simple logistic regression models were used to determine the association between biomarker expression (measured as a continuous variable) and response to pemetrexed therapy. Threshold analyses were completed for each biomarker. Wald χ² P values were plotted against ordered threshold values to determine the optimal expression threshold value that distinguished responders and nonresponders. A multiple regression model that included the binary form of TS, DHFR, and GARFT expression (measured as baseline expression by reverse transcription-PCR) and HER-2 (amplified versus nonamplified) was analyzed by backward stepwise elimination procedures (exit z = 0.1) to determine if these variables were predictive of chemotherapy response. Chemotherapy-associated changes in biomarker expression were evaluated as changes between baseline and post-baseline visits. One-sample t tests with z = 0.1 were used to determine if changes were different from zero. Simple logistic regression was used in post-hoc analyses to identify potential relationships between hormone receptor status and response to pemetrexed therapy.

Results

Patient characteristics

Between January 2001 and January 2002, a total of 76 patients entered the study at the Instituto de Enfermedades Neoplasicas in Lima, Peru. Fifteen patients were disqualified because they did not meet eligibility criteria (8 of 15) or withdrew informed consent (7 of 15). Baseline demographics and disease characteristics are summarized in Table 2. Sixty-one patients, with a median age of 46 years (range, 32-72 years), were enrolled. The vast majority of patients (59 of 61) had ductal breast cancer and 48% (29 of 61) of the tumors were estrogen receptor and/or progesterone receptor positive.

Clinical results

Treatment. A total of 176 pemetrexed doses were given to 61 patients. Fifty-seven patients (93.4%) received all three cycles of pemetrexed therapy and the median number of cycles delivered per patient was 2.9 (range, 1-3). Two patients received reduced doses of pemetrexed due to grade 2 diarrhea with dehydration (one of two) and study-site error (one of two). Although 60% of pemetrexed doses were delayed, the majority of these were due to scheduling conflicts, with a median delay of 5 days. Four patients discontinued therapy because of adverse events (two of four).

| Table 1. Primer sequences used in 5' nuclease assay (shown in the 5'→3' direction) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Primer name     | Forward sequence | Reverse sequence | TaqMan probe    | Primer name     | Forward sequence | Reverse sequence | TaqMan probe    |
| TS              | cctgatcatcactgcagcact | gaagaattcgacttcggagga | aattcagcttcagcagcagc | DHFR            | ggatagttggcagctgcttg | tgcagatcctgtctgacaatagtt | aagcccatgatcccccagccctgctt | GARFT           | gcctcccttttatacggtcagca | accgcagctgcacgctgccttg | tgtgccatgacgaagccctgga |

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On completion of pemetrexed therapy, 20 patients underwent excisional biopsy. No pathologic responses were observed.

**Efficacy.** Thirty-one percent (19 of 61) of patients achieved a partial tumor response after treatment with pemetrexed (Table 3). The majority of patients (56%) had stable disease. On completion of pemetrexed therapy, 20 patients underwent mastectomy (+/-- axillary node dissection) and 32 patients underwent excisional biopsy. No pathologic responses were observed.

**Toxicity.** Serious drug-related toxicities are summarized in Table 4. Elevated aspartate aminotransferase and alanine aminotransferase were both common, with grade 3 aspartate aminotransferase reported in 13 patients (21%) and grade 3/4 alanine aminotransferase reported in 20 patients (33%). In addition, five patients (8%) experienced grade 4 neutropenia and one patient reported grade 4 diarrhea. Other grade 3 nonlaboratory toxicities, including vomiting, nausea, fatigue, and headache, were generally rare (reported in two or fewer patients). Two patients discontinued therapy due to a serious episode of anxiety (one of two) that was unrelated to therapy and serious diarrhea and hypoproteinemia (one of two).

**Biomarker results**

*mRNA expression.* Tumor samples from 57 patients (18 responders and 39 nonresponders) were of sufficient quality for biomarker expression analysis. Responders had lower mean TS expression than nonresponders at all visits (Fig. 1); however, simple logistic regression (based on a 1-unit change of baseline expression of TS) indicated that this relationship was not statistically significant (odds ratio, 0.994; \( P = 0.103 \)). Baseline TS expression varied broadly among nonresponders (6-816 units on the relative scale for normalized TS mRNA expression) whereas the range of values among responders was ~4-fold smaller (22-207). Threshold analysis indicated that the TS expression value of 71 best distinguished responders and nonresponders. Thus, this value was used to categorize patients into binary expression groups ("high" versus "low"). The majority of responders (67%) were categorized as "low" baseline TS expressors (\( \leq 71 \)) whereas the majority of nonresponders (64%) had "high" baseline TS expression levels (\( > 71 \)).

For both DHFR and GARFT, responders exhibited higher baseline expression levels than nonresponders (Fig. 1), yet simple logistic regression analyses indicated that these relationships were not statistically significant (\( P = 0.311 \) for DHFR and \( P = 0.426 \) for GARFT). Baseline DHFR expression values ranged broadly (4-1,692) among responders and less broadly (3-176) among nonresponders. Similarly, baseline GARFT expression ranged broadly (8-1,199) among responders and less broadly (6-540) among nonresponders. Threshold analyses of marker expression yielded expression threshold values of 137 for DHFR and 17 for GARFT.

Multiple logistic regression analysis was done using clinical prognostic factors (age, performance status, and disease stage) and the expression level of selected molecular targets (TS, GARFT, and DHFR). However, this analysis did not identify statistically significant predictors of response. Post-hoc analyses did not show a relationship between baseline hormone receptor status and chemotherapy response.

Analysis of temporal shifts in biomarker expression among all patients allowed us to evaluate potential changes in tumor phenotype following pemetrexed exposure. Mean TS mRNA expression increased significantly between baseline and biopsy.

Table 3. Summary of best tumor response to pemetrexed therapy (N = 61)

<table>
<thead>
<tr>
<th>Overall study response</th>
<th>n (%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial response</td>
<td>19 (31)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>34 (55.7)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>6 (9.8)</td>
</tr>
<tr>
<td>Unknown*</td>
<td>2 (3.3)</td>
</tr>
</tbody>
</table>

*One patient, who withdrew after cycle 1, lacked follow-up assessments. One patient, who completed therapy, had one evaluable bone lesion assessed at baseline that was not assessed at later visits; assessments of measurable lesions for this patient, however, suggest that she had stable disease.
2 ($P = 0.004$) and subsequently decreased to near baseline levels at biopsy 3. Mean DHFR mRNA expression decreased between baseline and biopsy 2 ($P = 0.095$) and between baseline and biopsy 3 ($P = 0.092$). Similarly, mean GARFT expression decreased between baseline and biopsy 2 ($P = 0.006$) and between baseline and biopsy 3 ($P = 0.005$).

Subgroup analysis by response group revealed that non-responders (stable disease and progressive disease) had a significant increase in TS expression from baseline to biopsy 2 ($P = 0.016$). Nonresponders also showed significant decreases for both DHFR ($P = 0.001$) and GARFT ($P = 0.005$) from baseline to biopsy 2 and from baseline to biopsy 3 (DHFR, $P = 0.011$; GARFT, $P = 0.016$). Temporal shifts in TS, DHFR, and GARFT expression among the responder subgroup (Fig. 1) were not statistically significant ($P > 0.074$).

**HER-2 amplification status.** Fluorescence in situ hybridization experiments, done to determine if there was a significant relationship between HER-2 amplification status and response to pemetrexed, revealed no significant associations (data not shown).

**Discussion**

Primary chemotherapy provides an opportunity to downstage advanced disease in women with unresectable locally advanced or metastatic breast cancer, thus potentially allowing subsequent surgical treatment. Testing a new agent alone in the primary chemotherapy setting has the advantage of specifically isolating the activity of the drug without confounding factors that are associated with evaluating new agents as part of a combination regimen or within a pretreated population.

Potential ethical concerns exist, however, that delaying standard combination chemotherapy in this patient population by first treating patients with a potentially inactive, experimental drug might negatively affect patient outcomes or facilitate drug resistance (30). We are unaware of any data, however, that confirm that using a potentially ineffective single agent before standard therapy or briefly delaying the start of standard therapy shortens survival in patients with unresectable locally advanced or metastatic breast cancer. Experimental agents have been tested in this manner in recent studies of erlotinib (31), gefitinib (32), and lapatanib (33) in which standard therapies were delayed from 1 to 12 weeks. This approach is sound as long as patients are closely monitored for disease progression and adverse events.

Here we present data from a phase II trial and show single-agent activity of pemetrexed in advanced, previously untreated breast cancer patients with an overall response rate of 31%. The regimen has a manageable safety profile in advanced breast cancer and warrants further clinical development. This study also represents the first clinical pharmacogenomic report with pemetrexed in patients with advanced breast cancer. The present study suggests a potential association between “low”

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Grade 3 (%)</th>
<th>Grade 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutropenia</td>
<td>0</td>
<td>5 (8.2)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>1 (1.6)</td>
<td>0</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>13 (21.3)</td>
<td>0</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>19 (31.1)</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>2 (3.3)</td>
<td>0</td>
</tr>
<tr>
<td>Nausea</td>
<td>1 (1.6)</td>
<td>0</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1 (1.6)</td>
<td>0</td>
</tr>
<tr>
<td>Headache</td>
<td>1 (1.6)</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. mRNA expression of TS, DHFR, and GARFT for responder and nonresponder subgroups. Nonresponders were patients who had either stable disease or progressive disease. Biopsy 1 ($B_1$) was collected at baseline. Biopsy 2 ($B_2$) was collected 24 hours after infusion in cycle 1. Biopsy 3 ($B_3$) was collected after cycle 3. Vertical error bars, SD.
pretreatment TS expression and response to pemetrexed chemotherapy although this association did not reach statistical significance. Likewise, the majority of nonresponders (64%) had “high” TS expression before therapy. Such a relationship between pretreatment TS expression and tumor response was predicted by analyses of antifolate-resistant tumor cell lines in which reduced sensitivity to pemetrexed correlated with overexpression of TS (15, 34). Our observations are also consistent with findings from previous clinical studies conducted with 5-FU, which showed that tumor sensitivity to 5-FU was inversely related to pretreatment TS expression levels (16–18, 20).

Analysis of serial biopsy samples in the present trial revealed chemotherapy-associated changes in TS mRNA expression after pemetrexed treatment. We observed a significant increase in mRNA expression 24 hours after the first pemetrexed injection and a subsequent drop in TS expression at biopsy 3 (after the third cycle of pemetrexed therapy). To our knowledge, there has been no previous evidence of similar fluctuations in TS mRNA expression in patient tumors in response to treatment with antifolates or fluoropyrimidines. However, changes in TS protein levels in response to antifolate treatment in cell cultures have been described. Chu et al. (24) conducted experiments with human colon cancer cell lines and described an acute derepression of TS protein synthesis after short-term (8-36 hours) 5-FU exposure that did not result from increased TS mRNA. TS mRNA translation is known to be inhibited by its protein product through direct binding as part of an autoregulatory mechanism. Translational repression is relieved when TS binds one of its physiologic substrates or a direct inhibitor, resulting in increased synthesis of TS protein (35).

Unlike TS, the mean expression levels of both DHFR and GARFT were higher in pemetrexed responders than in nonresponders although this association was not statistically significant. This finding was unexpected given that increased expression of TS and DHFR generally correlates with decreased sensitivity to 5-FU and methotrexate, respectively (16–20, 36). With respect to GARFT, we are unaware of any studies that have examined the potential association between GARFT expression in tumors and chemosensitivity to GARFT-targeted agents. When expression in all patients was considered, we observed reduced mRNA levels for both DHFR and GARFT after pemetrexed treatment. The molecular determinants controlling the observed changes in TS, DHFR, and GARFT mRNA expression levels in breast tumors treated with pemetrexed are currently unknown.

In addition to the three genes that were analyzed in this study (TS, GARFT, and DHFR), other genes linked to antifolate resistance might be reasonable candidates for future studies designed to identify biomarkers of pemetrexed response. Certain cell lines have acquired antifolate resistance by modulating other cellular functions that control the uptake, retention, or activity of the respective agent. For example, antifolate resistance has been attributed to decreased antifolate polyglutamation resulting from decreased expression of polyglutamate synthetase (37–39). Polyglutamation normally increases the affinity of some antifolates for their targets, resulting in more potent inhibitors than parental compounds. Alterations in reduced folate carrier expression or activity have also been linked to reduced sensitivity to antifolates as a result of impaired antifolate uptake (40, 41). Future pharmacogenomic studies with pemetrexed should examine the modulation of these targets in response to therapy. A more comprehensive analysis of gene expression profiles would also be instructive.

In summary, data presented here suggest that lower pretreatment TS expression levels may be associated with enhanced clinical activity of pemetrexed. Our findings reinforce previous observations that TS is an important target for pemetrexed. The observed decreases in DHFR and GARFT expression after pemetrexed treatment highlight the potential clinical importance of these targets. Future translational research studies will include investigation of these biomarkers as well as additional genes important to the folate pathway and/or breast cancer. Such studies may ultimately allow us to identify biomarkers that, alone or in combination, are reliable predictors of response to pemetrexed.

Acknowledgments

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2. Ogston KN, Miller ID, Schofield AC, et al. Can mRNA levels for both DHFR and GARFT after pemetrexed in tumors and chemosensitivity to GARFT-targeted agents. When examined the potential association between GARFT expression With respect to GARFT, we are unaware of any studies that have

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In summary, data presented here suggest that lower pretreatment TS expression levels may be associated with enhanced clinical activity of pemetrexed. Our findings reinforce previous observations that TS is an important target for pemetrexed. The observed decreases in DHFR and GARFT expression after pemetrexed treatment highlight the potential clinical importance of these targets. Future translational research studies will include investigation of these biomarkers as well as additional genes important to the folate pathway and/or breast cancer. Such studies may ultimately allow us to identify biomarkers that, alone or in combination, are reliable predictors of response to pemetrexed.

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