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Pretreatment red blood cell total folate is associated with response to pemetrexed in stage IV non-squamous non-small-cell lung cancer

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Abstract

Objectives—Pemetrexed inhibits folate-dependent enzymes involved in pyrimidine and purine synthesis. Prior studies of genetic variation in these enzymes as predictors of pemetrexed efficacy have yielded inconsistent results. We investigated whether red blood cell (RBC) total folate, a phenotypic rather than genotypic marker of cellular folate status, was associated with response to pemetrexed-based chemotherapy in advanced non-squamous non-small-cell lung cancer (NSCLC).

Materials and methods—We conducted a prospective cohort study of patients with stage IV non-squamous NSCLC receiving first-line chemotherapy containing pemetrexed. Pretreatment RBC total folate was quantified using liquid chromatography/mass spectrometry. We then compared objective response rate (ORR) between patients with RBC total folate concentrations above and below an optimal cut-off value determined from the receiver operating characteristic (ROC) curve. A logistic regression model was used to adjust for age, sex, and use of bevacizumab.

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Conflicts of Interest: None

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Results—The ORR was 62% (32 of 52 patients). ROC analysis was used to establish that a RBC total folate cutoff value of 364.6 nM optimally discriminated between pemetrexed responders and non-responders. Patients with RBC total folate below 364.5 nM had an ORR of 27%, compared to 71% in patients with RBC total folate above this value ($p=0.01$). This difference persisted after adjusting for age, sex, and use of bevacizumab (OR 0.07, 95% CI 0.01 - 0.57, $p=0.01$).

Conclusions—Low pretreatment RBC total folate is associated with inferior response to pemetrexed-based chemotherapy in stage IV non-squamous NSCLC. Larger, multicenter studies are needed to validate RBC total folate as a predictive marker of pemetrexed response.

Keywords

chemotherapy; folic acid; biomarker; MTHFR; polymorphism

Introduction

Non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related mortality,¹ and most patients present with advanced stage disease. Despite recent advances in NSCLC therapy, cytotoxic chemotherapy with a platinum-based doublet remains the mainstay of first-line treatment.² While no randomized trial has demonstrated superiority for one platinum-based doublet over another for unselected advanced/metastatic patients, a pre-specified analysis of a phase III first-line study demonstrated superior overall survival (OS) with pemetrexed plus cisplatin compared to gemcitabine plus cisplatin in patients with non-squamous histology.³ Pemetrexed primarily targets thymidylate synthase (TS), a folate-dependent enzyme involved in DNA synthesis, DNA repair, and proliferation of cancer cells, as well as dihydrofolate reductase (DHFR) and glycylamide ribonucleotide formyltransferase (GARFT).^{4,5}

Although pemetrexed-based chemotherapy is the most commonly used regimen in patients with non-squamous histology, the reported objective response rate (ORR) is approximately 30%.^{3,6} Identification of predictive biomarkers other than non-squamous histology is needed to improve selection of patients who will derive the most benefit from pemetrexed-based chemotherapy. Intra-tumoral TS expression may be one marker of poor response to pemetrexed in NSCLC, as patients with TS-positive tumors had an inferior ORR and progression-free survival (PFS) in a recent randomized phase II trial.⁷ However, the practical utility of intra-tumoral TS expression is limited by the requirement for adequate tissue, as well as the subjective nature of current immunohistochemical approaches for assessment of TS expression and identifying an appropriate cutoff value to determine positivity. Genetic variation in cellular folate metabolism, specifically polymorphisms in *MTHFR* (encoding 5,10-methylenetetrahydrofolate reductase) and *TYMS* (encoding TS), may also impact tumor response to pemetrexed, though results of prior studies have been inconsistent.⁸⁻¹⁶

In light of the limitations of existing biomarkers, novel predictors of pemetrexed response are needed. We hypothesized that a blood-based phenotypic biomarker of folate status would be both practical and effective as a predictor of pemetrexed response, and accordingly investigated whether the pre-treatment red blood cell (RBC) total folate concentration is associated with response to pemetrexed-based chemotherapy in non-squamous NSCLC.

Materials and methods

Patients, Study Design, and Treatment

We conducted a single-center, prospective cohort study to determine whether pretreatment red blood cell (RBC) total folate was associated with response to pemetrexed-based chemotherapy in patients with newly diagnosed stage IV histologically-confirmed non-squamous non-small cell lung cancer (NSCLC). Eligibility criteria included use of pemetrexed and platinum containing regimen, measurable disease according to RECIST 1.1, and no prior systemic therapy for NSCLC. All patients were enrolled between September, 2010 and March, 2013 at the University of Pennsylvania and provided written informed consent. The study was approved by the Institutional Review Board of the University of Pennsylvania.

All patients received carboplatin (AUC 5-6) with pemetrexed 500mg/m² once every three weeks. Use of bevacizumab 15mg/kg, colony-stimulating factors and erythropoiesis-stimulating agents was allowed. Dose adjustment and cycle delays were allowed for resolution of toxicities. After four to six cycles of combination chemotherapy, the decision to continue with pemetrexed maintenance (with or without bevacizumab) was left to the discretion of the treating physician.

All patients received vitamin supplementation with 1mg oral folic acid daily and a 1000mcg intramuscular vitamin B12 injection given every nine weeks. A one-week run-in course of these vitamins was given to mitigate toxicity at, or soon after, the onset of chemotherapy. Baseline 25ml blood draws and the preparation of samples for measuring RBC total folate concentrations were performed prior to vitamin supplementation and chemotherapy administration. Treating physicians were blinded to the results of the patients' pre-treatment RBC total folate concentrations. Response to chemotherapy was assessed according to RECIST 1.1 by computed tomography (CT) scans on a schedule determined by the treating physician. All initial radiographic response assessments occurred after either the second or third cycle of chemotherapy. Treatment outcomes were categorized as partial response, stable disease, or progression. From these serial evaluations, the best outcome was used for the response rate evaluation. ORR was calculated as the percentage of partial responses among all treated patients.

RBC total folate measurement

Baseline RBC tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MTHF), and 5,10-methenyltetrahydrofolate (5,10-MTHF) levels were quantified as absolute nanomolar (nM) concentrations using a stable isotope dilution liquid chromatography, multiple reaction monitoring, mass spectrometry (LC-MRM/MS)-based method.¹⁷ This method permits the accurate quantification of folate analytes in RBCs.^{17,18} The RBC total folate concentration was calculated as the sum of these three analytes.

Genotype Analysis

In an exploratory analysis, we sought to determine whether the relationship between RBC total folate and pemetrexed response may be underpinned by polymorphisms in the 5,10-

methylenetetrahydrofolate reductase (*MTHFR*) and/or thymidylate synthase (*TYMS*) genes. DNA was extracted from blood samples and genotyped using a custom microarray based on Illumina BeadChip technology.¹⁹ We analyzed three exonic single nucleotide polymorphisms (SNPs) in the *MTHFR* gene [rs1801131 (1298A>C), rs1801133 (677C>T), and rs2274976 (1793G>A)], and two exonic SNPs in the 3'-untranslated region of the *TYMS* gene (rs2790 and rs699517). Each of these five SNPs was evaluated for an association with RBC total folate.

Statistical Analysis

All statistical analyses were performed using Stata version 14.1 (Stata, College Station, TX). Demographic characteristics were described using frequencies and percentages for categorical variables and medians and ranges for quantitative variables. The performance of pre-treatment RBC folate to discriminate between pemetrexed responders and non-responders was evaluated with the use of receiver operating characteristic (ROC) curves, and calculation of the area under the curve (AUC). The best cut-off level of RBC total folate to predict response was selected by using the Youden index, an objective method that maximizes the sum of sensitivity and specificity.²⁰ The chi-square test was used to determine the statistical significance (established at $p < 0.05$) of the association between RBC total folate concentration, as determined by the optimal cutoff point, and the primary clinical outcome of ORR. We repeated this analysis using logistic regression to adjust for age (categorized as less than 60 years, 60-69 years, and 70 years or older), sex, and use of bevacizumab concurrently with pemetrexed-based chemotherapy.

Secondary analyses included evaluation of the association of RBC total folate with progression-free survival (PFS) and overall survival (OS). PFS was defined as the number of months between the start of treatment and death or progression, whichever occurred first (or censored at date last seen). OS was defined as the number of months between the start of the treatment and death, or censored at the date of last patient contact. We calculated PFS and OS according to the Kaplan-Meier method and used Cox proportional-hazards models to adjust for age, sex, and bevacizumab administration.

To determine whether the difference in response rate according to RBC total folate concentration was attributable to absolute concentrations of one or more folate analytes or to relative concentrations of folate analytes, we used the Wilcoxon Rank-Sum test to compare concentrations of each analyte (5-MTHF, THF, and 5,10-MTHF) as well as the ratio of 5-MTHF to THF concentrations between responders and non-responders.

For genotype analysis, each of the five SNPs (dichotomized as homozygous for the more common allele versus heterozygous or homozygous for the less common allele) was evaluated for its association with RBC total folate using the Wilcoxon Rank-Sum test. The association of each SNP with response to pemetrexed was also assessed using Fisher's exact test. Bonferroni correction for multiple testing was undertaken, and $p < 0.01$ ($=0.05/5$) was set as the level of significance.

Results

Baseline characteristics and treatment

Baseline characteristics of the 52 study participants are described in Table 1. Pretreatment RBC total folate ranged from 118.6 to 2,553.1 nM (mean 693.4, median 560.3). All patients received pemetrexed with carboplatin, and 27 (52%) received bevacizumab concurrently. The ORR was 62% (32 of 52 patients), with all responses occurring prior to the fourth cycle of chemotherapy. Best responses for the remaining 20 patients included stable disease in 12 (23%) and progressive disease in 8 (15%). Of the 44 patients who achieved a response or had stable disease after three cycles, 17 (39%) went on to receive a total of four cycles, 5 (11%) received five cycles, and 22 (42%) received six cycles. Five patients progressed between cycles four and six of combination chemotherapy. Of the 39 patients whose disease had not progressed on combination chemotherapy, 29 (74%) went on to receive pemetrexed maintenance therapy (with or without bevacizumab).

RBC total folate and response to pemetrexed

A pre-treatment RBC total folate cutoff value of 364.6 nM optimally discriminated between pemetrexed responders and non-responders. The ROC curve including all possible cut points resulted in an AUC of 0.61 (95% CI 0.45 - 0.73). A total of 11 patients (21%) had pretreatment RBC total folate levels less than 364.6 nM, whereas 41 patients (79%) had concentrations above this cutoff. The ORR was 27% (3 of 11 patients) in the lower RBC total folate group (RBC total folate <364.6 nM) and 71% (29 of 41 patients) in the higher group ($p=0.01$). After adjusting for age, sex, and bevacizumab administration, there remained a significantly lower likelihood of response to pemetrexed for patients in the lower RBC total folate group (OR 0.07, 95% CI 0.01 – 0.57, $p=0.01$; Table 2).

We then assessed for differences in individual folate analyte levels between patients who responded to pemetrexed-based chemotherapy and those who did not. Median concentration of 5-MTHF was higher in pemetrexed responders compared to non-responders, though this did not reach statistical significance (537.8 nM vs. 379.1 nM, $p=0.06$). In contrast, there were no differences in the concentrations of THF ($p=0.61$), 5,10-MTHF ($p=0.37$), or the ratio of 5-MTHF to THF ($p=0.76$) between responders and non-responders (Figure 1).

Of the 31 patients with high RBC total folate who had a response or stable disease after four to six cycles of combined chemotherapy, 21 (68%) were treated with pemetrexed maintenance. The remaining 10 patients (32%) with high RBC total folate were either deemed unfit to continue with any maintenance ($n=1$) or were treated with bevacizumab maintenance alone (without pemetrexed) ($n=9$). All 8 patients (100%) with low RBC total folate who had a response or stable disease received pemetrexed maintenance.

Median PFS for the cohort was 6.3 months (95% CI 4.9-8.3 months), and median OS was 13.5 months (95% CI 9.7-19.5 months). Higher pretreatment RBC total folate was not associated with an improvement in PFS (adjusted HR 1.06, 95% CI 0.53 – 2.14, $p=0.87$) or OS (adjusted HR 1.00, 95% CI 0.47 – 2.14, $p=0.99$) after controlling for age, sex, and receipt of bevacizumab (Figure 2).

Analysis of *MTHFR* and *TYMS* variants with response to pemetrexed

DNA samples were available for genotyping for 42 of 52 patients (81%). A single variant (rs1801131; 1298A>C) in *MTHFR* that specifies a functional Glutamate to Alanine change at amino acid 429 was significantly associated with RBC total folate levels, with lower RBC total folate observed in patients with a homozygous CC or heterozygous AC genotype compared to homozygous AA patients ($p=0.005$; Figure 3). However, these genotypes did not reach statistical significance for association with pemetrexed response ($p=0.18$). No other variants considered in this analysis were associated with either RBC total folate concentration or pemetrexed response.

Discussion

Pemetrexed-based chemotherapy is widely used in advanced/metastatic non-squamous NSCLC, yet the response rate is only approximately 30-34%.^{3,6} Reliable predictive markers are needed to distinguish patients more likely to benefit from first-line pemetrexed-based chemotherapy from those who may be better served by initiating treatment with an alternative agent. In this prospective single-institution study, we found that pre-treatment RBC total folate concentrations were useful in predicting the probability of response to pemetrexed-based chemotherapy. To our knowledge, a blood-based predictive biomarker of pemetrexed response based on a phenotypic, rather than genotypic, marker of folate status has not previously been described.

Because we measured individual folate analyte concentrations, we were also able to evaluate whether the impact of RBC total folate on response to pemetrexed was attributable to absolute concentrations of one or more of the analytes or, alternatively, their relative concentrations. We found that the concentration of 5-MTHF, the major circulating form of folate and most prominent RBC analyte in most individuals,¹⁸ tended to be higher in pemetrexed responders compared to non-responders. Conversely, THF concentrations, 5,10-MTHF concentrations, and the ratio of 5-MTHF to THF concentrations were similar across both groups. Taken together, these findings suggest that it is the absolute concentration of RBC folate (primarily driven by 5-MTHF concentration), rather than the relative proportions of individual folate analytes, that is predictive of response to pemetrexed.

Although low RBC total folate concentration was not associated with inferior OS or PFS, this study was not powered to detect differences in survival. Furthermore, OS and PFS may have been influenced by subsequent therapies after progression on pemetrexed and by variation in the use of maintenance pemetrexed, respectively. For example, among patients with response or stable disease after 4-6 cycles of combination chemotherapy, all those with low RBC total folate received maintenance pemetrexed, compared to only two-thirds of those with high RBC total folate. Lastly, the relationship between response rate and OS in advanced NSCLC has not been firmly established,²¹ and it is possible that the difference in response rate predicted by RBC total folate may have no impact on survival.

Many previous studies have attempted to identify biomarkers associated with pemetrexed sensitivity. Because the histology-dependent differential efficacy of pemetrexed treatment *between* patients with non-squamous-cell and squamous-cell carcinoma correlates with

lower TS expression in the former tumor type,^{7,22-24} it has been hypothesized that lower tumor TS expression may serve as a predictive biomarker of increased pemetrexed sensitivity *within* the non-squamous NSCLC patient population.²⁵ This hypothesis was ultimately supported by a biomarker-stratified, randomized trial of non-squamous NSCLC patients that demonstrated increased clinical benefit from pemetrexed in TS-negative patients compared to TS-positive patients.⁷ However, the practical utility of intra-tumoral TS expression is limited by the need to acquire adequate tissue samples at biopsy or surgery, as well as a laboratory capable of properly processing such samples and quantifying and interpreting TS expression. The concentration of RBC total folate, the biomarker used in our study, can be easily determined using peripheral blood and can be measured by most hospital laboratories using commercial kits. If our findings are ultimately replicated in a larger study and show a survival differential, RBC total folate could readily be used as a predictive marker of response to pemetrexed.

Other studies aimed at identifying biomarkers to predict pemetrexed response have explored genetic variation in enzymes related to cellular folate metabolism, in particular *TYMS* polymorphisms. However, prior analyses of the relationship between such polymorphisms and outcomes in NSCLC patients treated with pemetrexed-containing regimens have had conflicting results. While several studies have shown that an increased number of 28-bp variable number tandem repeat (VNTR) sequences in the 5'-UTR of the *TYMS* gene (i.e. the 3R/3R genotype) and/or a 6-bp insertion/deletion polymorphism in the 3'-UTR of *TYMS* may influence outcomes with pemetrexed,^{10,11,13,15} others have demonstrated opposite results or no association at all.¹²⁻¹⁴ Similarly, two prior studies concluded that NSCLC patients who were homozygous for a substitution of thymine for cytosine in nucleotide 677 of the *MTHFR* gene (i.e. 677TT genotype) had improved PFS with second-line pemetrexed,^{9,16} while two subsequent studies failed to demonstrate any association between *MTHFR* genotypes and outcomes with pemetrexed.^{11,12}

There are numerous potential reasons that studies of genetic markers of pemetrexed response have shown inconsistent results. Most importantly, *TYMS* and *MTHFR* are only two of many enzymes, as well as receptors and transporters, that influence folate metabolism, and it is likely that polymorphisms in multiple genes contribute to differences in the effects of pemetrexed within a population. RBC total folate, the biomarker used in our study, serves as a key phenotypic, rather than genotypic, marker of folate metabolism. As such, the RBC concentration of total folate represents the net, measurable endpoint that results from the variable activities of multiple receptor, transporter, and enzyme polymorphisms.

Our exploratory genetic analysis indicated that the *MTHFR* 1298A>C polymorphism, a variant resulting in decreased efficiency of the MTHFR enzyme, is significantly associated with lower RBC total folate concentrations. This association makes sense, as decreased efficiency of MTHFR results in lower intracellular concentration of 5-MTHF, the key circulating form of folate and most prominent RBC folate analyte in the majority of individuals. The lack of a statistically-significant association between this polymorphism and response to pemetrexed may reflect the effects of multiple non-genetic and non-folate related influences on pemetrexed response, such as TS protein expression.⁷ However, it is interesting that the *MTHFR* 1298A>C polymorphism has been previously associated with

lower PFS in patients receiving second-line pemetrexed therapy for advanced NSCLC,⁹ as well as reduced OS in patients treated with pemetrexed for advanced gastric adenocarcinoma.²⁶ It is possible that our small sample size precluded detection of a PFS or OS difference according to this polymorphism, and larger studies will be needed to definitively assess the association of *MTHFR* 1298A>C, as well as *MTHFR* 677C>T, with RBC total folate and pemetrexed response.

The mechanism through which low baseline RBC total folate concentration was associated with inferior response to pemetrexed in our study is not known. The *MTHFR* 1298A>C polymorphism, a variant associated with decreased efficiency of the MTHFR enzyme and with lower RBC total folate in our study, may lead to higher intracellular concentrations of 5,10-methylenetetrahydrofolate, which competes with pemetrexed for interaction with the TS enzyme.⁸ It is therefore conceivable that higher concentrations of 5,10-methylenetetrahydrofolate result in increased competition with pemetrexed for inhibition of the TS enzyme, thereby lowering pemetrexed efficacy. However, separate studies are needed to formally test this hypothesis.

There are several limitations to our study. The sample size was small, as this study was designed to determine the feasibility of using RBC total folate as a biomarker of pemetrexed response and to derive an optimal RBC folate cutoff value. As a result, our study cohort included a high proportion of females and was younger than the general NSCLC population. In addition, the overall response rate was higher than expected based on prior phase III studies of pemetrexed-based chemotherapy. These differences from a typical NSCLC population may limit the generalizability of our findings. Lastly, we cannot rule out the possibility that low pretreatment RBC total folate is a biomarker for lower socioeconomic status and general nutritional health, both of which are potential confounders in the relationship between RBC total folate and response to pemetrexed-based chemotherapy. However, it seems unlikely that socioeconomic status and general nutritional health would lead to differences in objective radiographic tumor response, particularly without a difference in PFS or OS.

Conclusion

Low pre-treatment RBC total folate concentration is associated with an inferior response to pemetrexed-based chemotherapy in stage IV lung adenocarcinoma. Larger, multicenter studies are needed to validate RBC total folate as a predictive biomarker of pemetrexed in NSCLC.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015; 65(1):5–29. [PubMed: 25559415]

2. Shih C, Chen VJ, Gossett LS, et al. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. *Cancer Res.* 1997; 57(6):1116–1123. [PubMed: 9067281]
3. Scagliotti GV, Parikh P, von Pawel J, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naïve patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol.* 2008; 26(21):3543–3551. [PubMed: 18506025]
4. Shih C, Habeck LL, Mendelsohn LG, Chen VJ, Schultz RM. Multiple folate enzyme inhibition: mechanism of a novel pyrrolopyrimidine-based antifolate LY231514 (MTA). *Adv Enzyme Regul.* 1998; 38:135–152. [PubMed: 9762351]
5. Rhee MS, Ryan TJ, Galivan J. Glutamyl hydrolase and the multitargeted antifolate LY231514. *Cancer Chemother Pharmacol.* 1999; 44(5):427–432. [PubMed: 10501918]
6. Patel JD, Socinski MA, Garon EB, et al. PointBreak: a randomized phase III study of pemetrexed plus carboplatin and bevacizumab followed by maintenance pemetrexed and bevacizumab versus paclitaxel plus carboplatin and bevacizumab followed by maintenance bevacizumab in patients with stage IIIB or IV nonsquamous non-small-cell lung cancer. *J Clin Oncol.* 2013; 31(34):4349–4357. [PubMed: 24145346]
7. Sun JM, Ahn JS, Jung SH, et al. Pemetrexed Plus Cisplatin Versus Gemcitabine Plus Cisplatin According to Thymidylate Synthase Expression in Nonsquamous Non-Small-Cell Lung Cancer: A Biomarker-Stratified Randomized Phase II Trial. *J Clin Oncol.* 2015; 33(22):2450–2456. [PubMed: 26124486]
8. Chattopadhyay S, Moran RG, Goldman ID. Pemetrexed: biochemical and cellular pharmacology, mechanisms, and clinical applications. *Mol Cancer Ther.* 2007; 6(2):404–417. [PubMed: 17308042]
9. Smit EF, Burgers SA, Biesma B, et al. Randomized phase II and pharmacogenetic study of pemetrexed compared with pemetrexed plus carboplatin in pretreated patients with advanced non-small-cell lung cancer. *J Clin Oncol.* 2009; 27(12):2038–2045. [PubMed: 19307503]
10. Krawczyk P, Kucharczyk T, Kowalski DM, et al. Polymorphisms in TS, MTHFR and ERCC1 genes as predictive markers in first-line platinum and pemetrexed therapy in NSCLC patients. *J Cancer Res Clin Oncol.* 2014; 140(12):2047–2057. [PubMed: 25028118]
11. Li WJ, Jiang H, Fang XJ, et al. Polymorphisms in thymidylate synthase and reduced folate carrier () genes predict survival outcome in advanced non-small cell lung cancer patients treated with pemetrexed-based chemotherapy. *Oncol Lett.* 2013; 5(4):1165–1170. [PubMed: 23599757]
12. Kanazawa K, Yokouchi H, Wang X, et al. Phase II trial of carboplatin and pemetrexed as first-line chemotherapy for non-squamous non-small cell lung cancer, and correlation between the efficacy/toxicity and genetic polymorphisms associated with pemetrexed metabolism: Hokkaido Lung Cancer Clinical Study Group Trial (HOT) 0902. *Cancer Chemother Pharmacol.* 2014; 74(6):1149–1157. [PubMed: 25294632]
13. Hu Q, Li X, Su C, et al. Correlation between thymidylate synthase gene polymorphisms and efficacy of pemetrexed in advanced non-small cell lung cancer. *Exp Ther Med.* 2012; 4(6):1010–1016. [PubMed: 23226765]
14. Arevalo E, Castanon E, Lopez I, et al. Thymidylate synthase polymorphisms in genomic DNA as clinical outcome predictors in a European population of advanced non-small cell lung cancer patients receiving pemetrexed. *J Transl Med.* 2014; 12:98. [PubMed: 24726028]
15. Wang X, Wang Y, Wang Y, Cheng J, Wang Y, Ha M. Association of thymidylate synthase gene 3'-untranslated region polymorphism with sensitivity of non-small cell lung cancer to pemetrexed treatment: TS gene polymorphism and pemetrexed sensitivity in NSCLC. *J Biomed Sci.* 2013; 20:5. [PubMed: 23350714]
16. Tiseo M, Giovannetti E, Tibaldi C, et al. Pharmacogenetic study of patients with advanced non-small cell lung cancer (NSCLC) treated with second-line pemetrexed or pemetrexed-carboplatin. *Lung Cancer.* 2012; 78(1):92–99. [PubMed: 22889494]
17. Huang Y, Khartulyari S, Morales ME, et al. Quantification of key red blood cell folates from subjects with defined MTHFR 677C>T genotypes using stable isotope dilution liquid chromatography/mass spectrometry. *Rapid Commun Mass Spectrom.* 2008; 22(16):2403–2412. [PubMed: 18634122]

18. Mitchell LE, Morales M, Khartulyari S, et al. Folate and homocysteine phenotypes: Comparative findings using research and clinical laboratory data. *Clin Biochem.* 2009; 42(12):1275–1281. [PubMed: 19427846]
19. Baldwin DA, Sarnowski CP, Reddy SA, et al. Development of a genotyping microarray for studying the role of gene-environment interactions in risk for lung cancer. *J Biomol Tech.* 2013; 24(4):198–217. [PubMed: 24294113]
20. Youden WJ. Index for rating diagnostic tests. *Cancer.* 1950; 3(1):32–35. [PubMed: 15405679]
21. Blumenthal GM, Karuri SW, Zhang H, et al. Overall response rate, progression-free survival, and overall survival with targeted and standard therapies in advanced non-small-cell lung cancer: US Food and Drug Administration trial-level and patient-level analyses. *J Clin Oncol.* 2015; 33(9): 1008–1014. [PubMed: 25667291]
22. Ceppi P, Volante M, Saviozzi S, et al. Squamous cell carcinoma of the lung compared with other histotypes shows higher messenger RNA and protein levels for thymidylate synthase. *Cancer.* 2006; 107(7):1589–1596. [PubMed: 16955506]
23. Sun JM, Han J, Ahn JS, Park K, Ahn MJ. Significance of thymidylate synthase and thyroid transcription factor 1 expression in patients with nonsquamous non-small cell lung cancer treated with pemetrexed-based chemotherapy. *J Thorac Oncol.* 2011; 6(8):1392–1399. [PubMed: 21716147]
24. Tanaka F, Wada H, Fukui Y, Fukushima M. Thymidylate synthase (TS) gene expression in primary lung cancer patients: a large-scale study in Japanese population. *Ann Oncol.* 2011; 22(8):1791–1797. [PubMed: 21321092]
25. Liu Y, Yin TJ, Zhou R, Zhou S, Fan L, Zhang RG. Expression of thymidylate synthase predicts clinical outcomes of pemetrexed-containing chemotherapy for non-small-cell lung cancer: a systemic review and meta-analysis. *Cancer Chemother Pharmacol.* 2013; 72(5):1125–1132. [PubMed: 24067998]
26. Chen JS, Chao Y, Bang YJ, et al. A phase I/II and pharmacogenomic study of pemetrexed and cisplatin in patients with unresectable, advanced gastric carcinoma. *Anticancer Drugs.* 2010; 21(8):777–78. [PubMed: 20634689]

Clinical Practice Points

- Pemetrexed is used extensively in non-squamous NSCLC without a reliable biomarker of response
- Low pretreatment RBC total folate was associated with lack of response to first-line pemetrexed plus platinum in advanced non-squamous NSCLC
- Our study suggests potential use of RBC total folate, a low cost and widely available test, for predicting pemetrexed response

Figure 1. Distribution (log scale) of pretreatment red blood cell (RBC) folate analyte concentrations (nM) in pemetrexed responders (n=32) and non-responders (n=20)
 (A). 5-methyltetrahydrofolate (5-MTHF) (B). 5,10-methenyltetrahydrofolate (5,10-MTHF)
 (C). tetrahydrofolate (THF) (D). Ratio of 5-MTHF:THF

Figure 2. Effect of red blood cell (RBC) total folate on overall and progression-free survival
(A) Progression-free survival (adjusted HR 1.1, 95% CI 0.5 – 2.1) and (B) Overall survival
(adjusted HR 1.0, 95% CI 0.5 – 2.1)

Figure 3. Effect of the MTHFR 1298A>C polymorphism on red blood cell (RBC) total folate concentration (log scale)

Patients with the 1298AA genotype had higher RBC total folate than patients with the 1298AC or 1298CC genotypes

Table 1
Baseline Clinical Characteristics

Characteristic	N=52
Sex – no. (%)	
Female	27 (52)
Male	25 (48)
Race – no. (%)	
White	43 (83)
Black	8 (15)
Not reported	1 (2)
Age – years	
Median	62
Range	35-83
Ever Smoker – no. (%)	46 (88)
ECOG performance status – no. (%)	
0	12 (23)
1	34 (65)
2	6 (12)
Oncogenic driver mutation – no. (%)	
EGFR mutation	0 (0)
ALK translocation	1 (2)
ROS1 translocation	1 (2)
Concurrent bevacizumab – no. (%)	27 (52)

Table 2
Multivariate analysis of effect of RBC total folate on objective response rate to pemetrexed

Variable	Odds ratio for response	95% confidence interval	p-value
Red blood cell total folate (low/high)	0.07	0.01 – 0.57	0.01
Age – years			
<60	1.00		
60-69	2.17	0.33 - 14.17	0.42
70	0.35	0.06 - 2.18	0.26
Sex (female/male)	1.34	0.34 - 5.24	0.68
Bevacizumab (+/-)	7.00	1.53 - 31.98	0.01