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Pralatrexate in Combination with Oxaliplatin in Advanced Esophagogastric Cancer: A Phase II Trial with Predictive Molecular Correlates

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ABSTRACT

The purpose of our study was to evaluate the efficacy of a combination of pralatrexate plus oxaliplatin in advanced esophagogastric cancer (EGC), analyze the impact of polymorphisms in folate metabolism pathway genes on toxicity and efficacy of pralatrexate, and to evaluate microRNA profile of tumor epithelium as a predictive biomarker. This was a two-stage trial with a safety lead in cohort and a primary endpoint of overall response rate (ORR). Patients received biweekly intravenous oxaliplatin (85 mg/m^2) and pralatrexate (Dose level 1 [D1], 120 mg/m^2; dose level-1 [D-1] 100 mg/m^2). Single-nucleotide polymorphisms (SNP) in genes encoding proteins involved in pralatrexate metabolism were evaluated in germline DNA. microRNA profiling of the tumor epithelium was performed. ORR was 26%. Dose-limiting toxicities were observed in 2 of 4 patients at D1 and none at D-1. The *T*>*C* polymorphism in *DHFR* rs11951910 was significantly associated with lower progression-free survival (PFS; $P \leq 0.01$), whereas the presence of the *SLC19A1* rs2838957 *G*>*A* polymorphism was associated with improved PFS ($P = 0.02$). Presence of the *GGH* rs3780130 *A*>*T* and *SLC19A1* rs1051266 *G*>*A* polymorphisms were significantly associated with better overall survival (OS; $P = 0.01$), whereas *GGH* rs7010484 *T*>*C* polymorphism was associated significantly with reduced OS ($P = 0.04$). There was no correlation between epithelial microRNA expression profile with disease progression or response. We conclude that the combination of oxaliplatin and pralatrexate is safe, is well tolerated, and has modest efficacy in advanced EGC. Pharmacogenomic analysis may be relevant to the use of pralatrexate in combination with platinum agents.

Introduction

Esophageal and gastric cancers are highly aggressive malignancies. There has been a dramatic increase in the incidence of esophageal adenocarcinoma in the United States in the past two decades with a concurrent 7-fold increase in mortality (1). There is no uniform global standard for the first-line therapy of advanced esophagogastric cancer (EGC). Regimens include doublet or triplet-combination chemotherapy agents, usually platinum- or 5-fluorouracil (5FU)–based, with response rates ranging from 15% to 50% (2). Although both the addition of trastuzumab in HER-2/neu-overexpressing tumors and the use of ramucirumab in second line treatment improve survival in subgroups of patients, the five-year survival for advanced EGC remains dismal at 10% to 20% (3).

Oxaliplatin is a favorable alternative to cisplatin, with improved toxicity and preserved efficacy (4). It is less emetogenic, less nephrotoxic, and less neurotoxic compared with cisplatin. As such, oxaliplatin has been widely incorporated into combination treatment regimens in advanced EGC (5–10). Our group has systematically demonstrated the feasibility and efficacy of oxaliplatin in combination with 5FU, and with capecitabine in three successive trials in esophagus cancer, all in combination with radiotherapy (11, 12). This regimen achieved a pathologic complete response rate of 33% for resectable esophagus cancer in SWOG 0356, a multicenter phase II trial. Major drawbacks of the oxaliplatin plus 5FU doublets are inconvenience of the 5FU infusion schedule and the need for central venous access and infusion pump. Hence, it is imperative to investigate incorporation of novel agents in platinum-based combinations.

The anti-folate drugs methotrexate and pemetrexed have demonstrated activity in EGC (13–16). Pemetrexed has shown greater efficacy compared with 5FU against several gastric cancer cell lines (17). A phase II trial assessing pemetrexed (500 mg/m^2) in combination with oxaliplatin (120 mg/m^2) every 21 days in advanced gastric cancer demonstrated a response rate of 36%, with four complete responses and a notable time-to-progression of 6.2 months (18). Similar combinations have been investigated in non–small cell lung cancer (19). Pralatrexate is an analogue of methotrexate and acts as a potent dihydrofolate reductase (DHFR) inhibitor. Studies indicate that pralatrexate selectively enters cells expressing reduced folate carrier (RFC-1) that is encoded by the gene *SLC19A1*; and its increased retention in cells results from its high affinity for folylpolyglutamate synthase protein (FPGS). Like pemetrexed, the steady-state accumulation of

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pralatrexate polyglutamates depends primarily on activation/inactivation processes by FPGS and gamma glutamyl hydrolase (GGH), respectively (20–22). In previous studies with pemetrexed in NSCLC, we identified correlations between haplotype-tagged single-nucleotide polymorphisms (ht-SNP) in *SLC19A1, GGH*, and *FPGS* and efficacy as well as toxicity (23, 24) Pralatrexate has demonstrated synergy with platinum and taxane compounds (25, 26). The documented preclinical synergy with platinum makes it an ideal candidate for testing in a combination regimen. Therefore, we sought to evaluate the antitumor efficacy of pralatrexate administered every other week in combination with the standard dose of biweekly oxaliplatin in patients with advanced EGC. Furthermore, we evaluated whether functionally relevant germline polymorphisms in selected genes of the folate pathway (*DHFR, FPGS, GGH, SLC19A1, ATIC, GART, SLC46A1*, and *TYMS*) as well as a tumor microRNA (miRNA) signature can serve as predictive biomarkers for efficacy and or toxicity.

Materials and Methods

Study design

We designed a two-stage, phase II clinical trial of oxaliplatin in combination with pralatrexate in patients with advanced (metastatic or locally advanced) EGC who had not received any prior systemic therapy in the advanced setting. As there were no safety data for this specific combination, the combination was tested first in a lead in design in 6 patients with pralatrexate dose de-escalation in the event of dose-limiting toxicity (DLT). A dose level was considered tolerable if following completion of two cycles (28 days) of treatment, 5 out of 6 patients did not develop DLT. DLT was defined as any of the following conditions related to study treatment occurring within the first 28 days of treatment (2 cycles): (i) grade 4 neutropenia lasting >7 days, (ii) febrile neutropenia, defined as grade 3 or 4 neutropenia associated with a fever of >100.4°F (=38.0°C) or higher, (iii) grade 4 thrombocytopenia (iv) any grade 3 or higher non-hematologic toxicity except grade 3 diarrhea, nausea or vomiting in the absence of optimal anti-diarrheal or anti-emetic treatment and grade 3 hypocalcemia, hypokalemia, hypomagnesemia, hyponatremia, or hypophosphatemia which responds to medical intervention (provided the toxicity resolved within 7 days without need for dose reduction), and (v) treatment delay of >14 days for toxicity. Patient tissue and blood samples were collected prospectively for correlative studies (genotyping for SNPs in folate metabolism genes and miRNA profiling). CONSORT guidelines were used for the study (ClinicalTrials.gov number, NCT01178944).

Study population

Patients with histologically confirmed carcinoma of the esophagus, stomach or gastro–esophageal junction (EGC) who were deemed metastatic or inoperable for cure were eligible. Additional eligibility criteria included no prior systemic therapy for advanced disease, an ECOG performance status if 0–2, adequate hematological (hemoglobin >9 g/dl, absolute neutrophil count >1,500/mm^3, platelet count >100,000/mm^3) and biochemical parameters (serum creatinine < institutional upper limit normal (ULN), bilirubin < 1.5 X ULN, transaminases < 3 X ULN; for documented liver metastases, transaminases up to 5 X ULN is permitted), absence of $\geq$ grade 2 peripheral neuropathy. Patients with brain metastases and presence of third-space (pleural, peritoneal) fluid not controllable with usual drainage methods were excluded. All patients were required to sign an IRB approved written informed consent per institutional guidelines.

Study objectives and endpoints

Objectives

Our primary objective was to determine the overall response rate (ORR) to combination pralatrexate and oxaliplatin in patients with advanced EGC. Our secondary objectives were to examine the toxicity and tolerability of this regimen and the time-to-progression and overall survival (OS) using this regimen. We also wanted to examine whether functionally relevant polymorphisms of genes in the folate metabolism pathway correlate with efficacy and toxicity of pralatrexate. Finally, we aimed to assess whether response to pralatrexate can be predicted by microRNA expression profiling of the epithelial component of the tumor.

Endpoints

The primary end-point of this phase II trial was the ORR determined by using the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1. The toxicity of this regimen was recorded for all patients who received at least one dose of therapy. Other end-points included calculation of time-to progression and OS.

Study treatment and procedures

Following antiemetic therapy with HT3 antagonist and dexamethasone, pralatrexate was administered intravenously over 3–5 minutes followed by oxaliplatin at 85 mg/m^2 intravenously over 2 hours. The starting dose for pralatrexate was 120 mg/m^2. Dose level-1 was 100 mg/m^2. Oxaliplatin was stopped after 12 cycles (maximum cumulative dose = 1,020 mg/m^2) to reduce the risk of peripheral neuropathy. Treatment was repeated every 2 weeks until disease progression, undue toxicity, or patient/physician discretion.

All patients received standard anti-emetic and supportive therapy. Folic acid (1–1.25 mg/d) was given orally daily starting at least 10 days before the first dose of pralatrexate. Folate was continued during the full course of treatment with pralatrexate and for 30 days after the last dose of pralatrexate. Vitamin B 12 (1,000 mcg) was administered intramuscularly no more than 10 weeks before the first dose of pralatrexate and repeated every 8 to 10 weeks thereafter while on treatment with pralatrexate.

Specimens for correlative studies (peripheral blood mononuclear cells-PBMCs and archival tumor tissue) were collected at baseline. Although on therapy patients were evaluated every 2 weeks for toxicity monitoring and restaging cross sectional imaging was obtained after 4 cycles or 8 weeks of therapy. Patients who discontinued therapy were followed at 30 days to assess resolution of toxicities and then per standard of care for a maximum period of 5 years or until death for survival assessment.

Correlative studies

Pharmacogenomic analysis

Germline DNA from PBMCs collected at baseline were analyzed for polymorphisms in genes involved in the metabolism of pralatrexate. These genes included *DHFR, FPGS, GGH, ATIC, GART, SLC19A1, SLC46A1*, and *TYMS* that activate, inactivate, transport and are targets for pralatrexate. TagSNPs were derived similarly as described previously (27) and the DNA samples were genotyped for the SNPs at the Microarray and Genomics facility at Roswell Park Cancer Institute using the MassARRAY Compact system (Sequenom).

Tumor microRNA analysis

Formalin-fixed and paraffin-embedded tissue sections of 8-μm thickness were placed on glass slides covered with polyethylene naphthalate membrane (Leica). After de-paraffinization with xylene

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and rehydration with a graded ethanol series, the slides were stained with hematoxylin and eosin Y. Slides were then dehydrated using a reverse graded ethanol series and xylene, and used for laser capture microdissection (LCM) within a day. LCM to collect cancerous epithelial component of tissue sections was performed under the guidance of an experienced pathologist with an ultraviolet laser on an LMD6000 system (Leica) at ×50 and ×200 magnification and under >35% humidity.

Before RNA isolation, microdissectates (**Fig. 1**) were stored at −80°C for up to a month in Digestion Buffer A provided with FFPE RNA Purification kit (product number 25300; Norgen Biotek). Total RNA isolation, which included steps for treatments with proteinase K and DNAse I, was performed as per the manufacturer's guidelines. Between 0.12 and 2.36 μg of RNA (mean 1.01, SD 0.56) was obtained from microdissected epithelial components for each tumor. Ten-ng RNA was poly-adenylated and reverse transcribed using Universal cDNA Synthesis II kit (product number 203301, Exiqon). PCRs of 15 μL volume to amplify specific small RNAs were performed in a 384-well plate on a LightCycler 480 instrument (Roche). Before hybridization to a microarray, 0.35 μg of a human reference RNA (Ambion product number AM6000) and a microdissectate RNA sample were, respectively, 5′- and 3′-end-labeled with Hy5 and Hy3 fluorophores using miRCURY LNA microRNA Hi-Power Labeling kit (Exiqon). For the microdissectate RNAs, 0.35 μg was used for 25 microarrays, and between 0.11 and 0.30 μg for the rest of 4 samples with lower RNA yield due to limited tissue. For assessment of technical replicability, one microdissectate RNA was assayed with a total of four microarrays, using duplicate pairs of 0.35 or 0.28 μg RNA. Microarrays were scanned on G2565BA Microarray Scanner System (Agilent) for image analysis with ImaGene software (version 9; BioDiscovery). Correction for background noise was done using the normexp method (27) with an "offset" value of 10, and was followed by within-array normalization using the global loess regression method with a "span" value of 1/3 (28). Inter-sample normalization of microarray signal values was then performed with the quantile method. Finally, signal values were identified as summarized Hy3 values that were the means of values from the quadruplicate probe-spots if the maximum was <1.5 times the minimum, or the medians if otherwise. RNAs recognized by probes with signal values were >3 times the summarized signal value for probe-less empty microarray spots (1,132 total) in at least a quarter of the 32 microarray assays that were performed were considered as expressed.

Statistical analysis

The study was an exact two-stage, single arm Phase II trial allowing for early termination in case of futility. The primary end point of the study was ORR, defined as complete or partial response. In the standard-of-care therapy, ORR for advanced EGC was reported around 0.20. The following study design had 80% power to detect a 0.40 ORR with the study treatment, while controlling to 5% the probability of erroneously finding a truly ineffective treatment as worthy of further research. Seventeen eligible patients (including the safety lead in phase) were required to be enrolled in the first stage of the trial. If a maximum of 3 of these patients achieved complete or partial response, the treatment was concluded to be ineffective, and the study terminated. If 4 or more patients achieved complete or partial response, an additional 16 patients were to be enrolled. If 11 or more of the 33 total patients achieved complete or partial response, the treatment was to be deemed effective and considered for further study.

Progression-free survival (PFS) was measured from the date of study enrollment to the first observation of progressive disease. OS was measured from the date of study enrollment to the time of death from any cause. Statistics describing the time to event distributions were obtained from Kaplan–Meier methods and Proportional Hazards

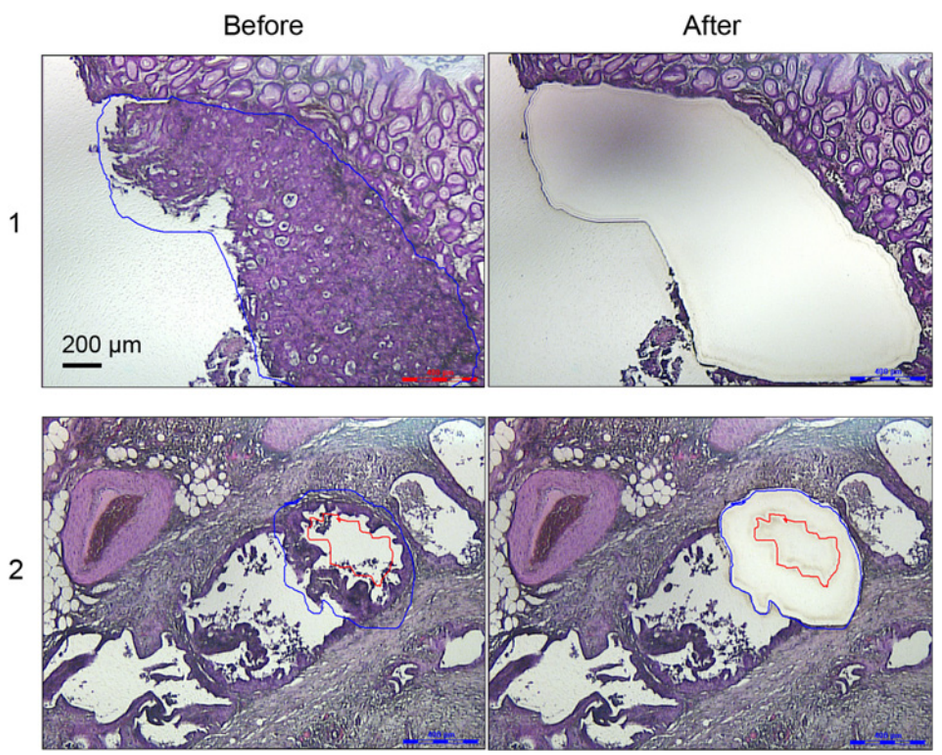

Figure 1.
Representative microscopic images of tissue sections taken before and after dissection of cancerous epithelial components.

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models. Continuous variables are summarized with commonly used statistics (mean, standard deviation, median etc.), with sub-group associations tested using the Wilcoxon Rank Sum test. Categorical variables were summarized in contingency Tables, with associations of interest assessed using Fisher's Exact test. All *P* values were 2-sided and a value of less than 0.05 was deemed statistically significant.

Patient genotypes were correlated in an exploratory analysis with OS and PFS. Logistic regression models were used to compare the ORR between the different SNP subgroups. Kaplan–Meier curves were used to visually compare the OS and PFS distributions between the different SNP subgroups, and Cox regression models were used to assess the impact of the genotype subgroups on OS and PFS.

Log_2-transformed normalized microarray signal values for 29 cases for the 1,031 microRNAs that were identified as expressed in the study were used for all analyses. For comparison of microRNA measurements between two groups, empirical Bayes-moderated t statistics as implemented in the limma Bioconductor package were used. *P* values were adjusted for multiple testing to control false discovery with the Benjamini–Hochberg method. The dist, hclust, and prcomp functions of R were used for clustering and principal component analyses. Euclidean distances and complete linkages were used for clustering.

Results

Safety lead-in (cohort 1)

Patients were enrolled in cohort 1 from December 2010 to November 2011 at a single institution. Baseline characteristics are presented in **Table 1**. Four patients were treated at dose level 1 (120 mg/m^2) of Pralatrexate. Two developed dose-limiting toxicities (DLT). One patient had grade 3 mucositis and a second patient had grade 3 diarrhea. Pralatrexate was reduced to dose level-1 (100 mg/m^2) for all remaining subjects. Six additional patients were enrolled at dose level-1 and no further DLTs were observed. As such dose level-1 was considered the recommended phase II dose.

Table 1. Patient characteristics.

	Dose level-1 (D-1)	Dose level 1 (D1)	Overall
N	31 (88.6)	4 (11.4)	35 (100%)
Age, y			
Mean/std	64.9/10.2	63.6/7.5	64.7/9.9
Median/min/max	67.0/39.2/82.5	64.7/54.0/71.0	67.0/39.2/82.5
Gender			
Male	29 (93.5%)	3 (75.0%)	32 (91.4%)
Female	2 (6.5%)	1 (25.0%)	3 (8.6%)
Race			
White	31 (100.0%)	3 (75.0%)	34 (97.1%)
Black, African American		1 (25.0%)	1 (2.9%)
ECOG			
0	14 (45.2%)	1 (25.0%)	15 (42.9%)
1	15 (48.4%)	3 (75.0%)	18 (51.4%)
2	2 (6.5%)		2 (5.7%)
Site			
Esophagus	25 (80.6%)	4 (100.0%)	29 (83%)
Stomach	6 (19.4%)		6 (17%)
Histology			
Squamous cell	2 (6.5%)	1 (25.0%)	3 (8.6%)
Adenocarcinoma	26 (83.9%)	3 (75.0%)	29 (82.9%)
Poorly differentiated	1 (3.2%)		1 (2.9%)
Adenosquamous	2 (6.5%)		2 (5.7%)
Clinical stage			
II	1 (3.2%)		1 (2.9%)
III	1 (3.2%)		1 (2.9%)
IV	25 (80.6%)	4 (100.0%)	29 (82.9%)
MISC	4 (12.9%)		4 (11.4%)
Completed cycles			
Mean/std error	7.4/0.8	5.5/2.2	7.2/0.7
Median/min/max	8.0/1.0/17.0	4.0/2.0/12.0	6.0/1.0/17.0

Phase II

Demographics and baseline characteristics

A total of 31 patients were treated at dose level-1 from January 2012 to February 2015. No DLTs were observed in the phase II part of the study. Demographics and baseline characteristics are summarized in **Table 1**. As anticipated, a majority of the patients were male (91.4%) and Caucasian (97.1%). Median age was 67 years with adenocarcinoma being the predominant histology (82.9%). The median number of Pralatrexate cycles received at dose level D-1 was 8.

Efficacy

Out of all 35 patients, 3 patients came off study after initiation of cycle 1, before collection of first-response data and were considered not evaluable. One patient had dose limiting toxicity with grade 3 mucositis at dose level D1, another patient declined any further therapy and a third patient required radiotherapy for painful skeletal metastases. These patients were treated as non-responders. All 35 patients were included in toxicity evaluation and final survival analysis (**Fig. 2**).

Primary endpoint

Partial response was seen in 9 patients. No patient achieved a complete response. As the design required 11 responses for the treatment to be considered effective, the study did not achieve the defined primary end point of efficacy. Excluding the three patients deemed unevaluable, the ORR was 28% [95% confidence interval (CI), 14–47]. An ORR of 26% (95% CI, 12–43) was observed in the 35 patients who received at least one cycle of study (**Table 2**).

Secondary endpoints

All 35 patients who received at least one cycle of study treatment were included in survival analysis. Using Kaplan–Meir curve, PFS was estimated at 5.1 months (95% CI, 3.4–6.4) and OS was 7.2 months (95% CI, 6.4–10.8; **Table 3**; **Fig. 3A** and **B**). Because the historical median OS in this population is 10 months, we performed an exploratory analysis to see if the long-term survivors (patients living beyond the expected median OS of 10 months) had longer duration of treatment. We found that there was no difference in the number of cycles ($P = 0.08$) in patients with OS <10 vs. >10 months. Similarly, the PS, albumin level, neutrophil/lymphocyte ratio and LDH level were also similar between patients with OS <10 vs. >10 months (Supplementary Table S4).

Safety

Two of the first 4 patients at D1 developed DLTs (One G3 diarrhea, One G3 mucositis). All remaining pts were treated at D-1 (no DLTs in the safety cohort at this level). G4 toxicity included neutropenia ($N = 2$, 6%) and myocardial infarction ($N = 1$, 3%). The most common G_3 events were mucositis ($N = 5$, 14%), dehydration and neutropenia ($N = 4$, 11% for each), anorexia, neuropathy, fatigue and nausea/vomiting ($N = 3$, 9% for each). Including G_1 and $_2$ events, mucositis

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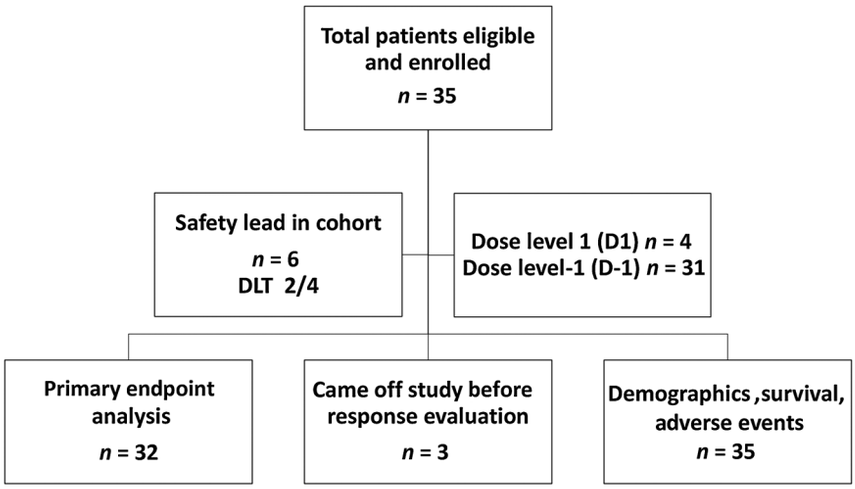

Figure 2.
Patient enrollment, treatment allocation with different dose levels, and how they were analyzed in the trial.

($N = 30$, 86%), anemia ($N = 27$, 77%) and neuropathy ($N = 27$, 77%) were the most common toxicities. Overall the toxicities were manageable and therapy well tolerated at Dose level-1 (Supplementary Tables S1 and S2). We wanted to examine whether certain phenotypes are associated with increased toxicity. Supplementary Table S6 illustrates polymorphisms of the ATIC gene (a gene involved in folate pathway and purine biosynthesis) that are associated with increased grade 3 or higher toxicity related to Pralatrexate.

Correlative studies

SNPs in folate metabolism genes

Fifty-seven tagSNPs from the 8 genes were genotyped in all 35 patients enrolled in the study. Two SNPs were homozygous in all samples, 2 failed genotyping and 5 SNPs were not in Hardy–Weinberg equilibrium leaving a total of 48 SNPs for analysis with clinical outcomes, Supplementary Table S3. Variant genotypes observed in less than 4 (10%) patients were regrouped and if the regrouped frequency was $\leq$10%, the SNP was excluded from the analyses with the clinical outcomes. The polymorphisms with significant associations are shown in **Table 4**. *DHFR* rs11951910 and *SLC19A1* rs2838957 were associated with PFS whereas *GGH* rs3780130 and rs7010484 and *SLC19A1* rs1051266 were associated with OS. The presence of the variant allele in *DHFR* rs11951910 *T>C* was associated significantly with lower PFS (*TT* vs. [*TC+CC*]: 5.6 vs. 2.9 months; log-rank test $P \leq 0.01$) whereas the *SLC19A1* rs2838957 *G>A* polymorphism was significantly associated with an improved PFS *(GG* vs. *[GA+ AA]:* 3.7 vs. 5.6 months; log-rank test *P = 0.02)*. On the other hand, the presence of the variant allele in *GGH* rs3780130 *A>T* showed a significant association with a better OS *(AA* vs. *[AT+TT]:* 6.8 vs. 11.4 months; log-rank test *P = 0.01)* as did the polymorphism in *SLC19A1* rs1051266 *G>A (GG* vs. *[GA+AA]:* 6.7 vs. 10.1 months; log-rank test *P = 0.01)*. Finally, the presence of the variant allele in *GGH* rs7010484 *T>C* was associated with reduced OS *(TT* vs. [*TC+CC*]; 9.2 vs. 6.9 months; log-rank test *P = 0.04)*. We found that certain SNPs correlate with number of cycles of drug as illustrated in Supplementary Table S5; however, those SNPs were not associated with OS.

Tumor microRNA expression

We examined whether disease progression was associated with microRNA expression levels in cancer epithelia. Adequate tumor epithelia was available for analysis in 29/35 cases. Whereas 350 ng RNA was used for microarray-based global microRNA profiling for the vast majority of the 29 cases, the amount of RNA was 110–300 ng for four cases because of inadequate RNA yield from microdissected tissue. MicroRNA measurements obtained with the platform demonstrated good reproducibility. Measurements for a total of 1,956 microRNAs were obtained, and 1,032 microRNAs were identified as expressed among the 29 cases. The microarray data were partially validated by examining correlations of measurements of six RNAs for 12 cases with measurements obtained by RT-PCR. Pearson correlation coefficients were >0.85 for all six RNAs.

To assess association of microRNA expression with disease progression, we evaluated the microRNA profiles in three different ways. Principal component analysis of microRNA data did not reveal profile similarity by disease progression. Unsupervised hierarchical clustering too did not indicate any similarity of microRNA expression among cases grouped by disease progression (Supplementary Fig. SF1). Finally, in differential expression analyses for comparing two groups, expression of no microRNA was identified as being significantly different ($P > 0.05$) between the group of cases with disease progression ($n = 7$), and either the group for stable disease ($n = 14$), or the group for partial response or remission ($n = 6$). The result was similarly

Table 2. Treatment outcome.

	D-1	D1	Overall
NE, not evaluable	2 (6.5%)	1 (25.0%)	3 (8.6%)
PD, progressive disease	8 (25.8%)	1 (25.0%)	9 (25.7%)
ORR (partial response/remission)	8 (25.8%)	1 (25.0%)	9 (25.7%)
SD, stable disease	13 (41.9%)	1 (25.0%)	14 (40.0%)

Table 3. Survival analysis.

Survival	6-Month survival rate (95% CI)	1-Year survival rate (95% CI)	Median survival in months (95% CI)
Progression-free survival	0.40 (0.24–0.56)	0.17 (0.07–0.31)	5.1 (3.4–6.4)
Overall survival	0.69 (0.50–0.81)	0.31 (0.17–0.47)	7.2 (6.4–10.8)

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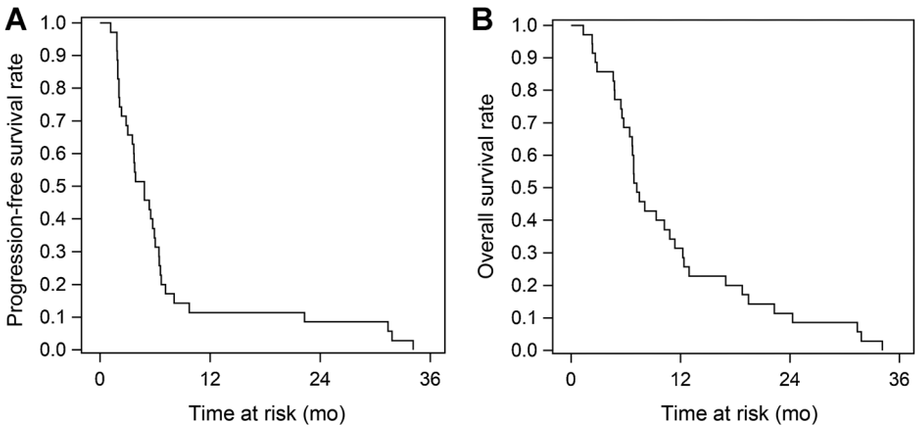

Figure 3.
A, Unadjusted PFS; **B,** Unadjusted OS.

negative in analysis in which the latter two groups were combined. No differential microRNA expression was observed in similar comparisons in analyses of only adenocarcinoma cases. Thus, an association between tumor epithelial microRNA expression and disease progression could not be identified. Expected differences in microRNA expression between squamous cell carcinoma and adenocarcinoma epithelia, for example, for miR-192-5p, -194-5p and -205-5p (29), were noticeable in the microRNA data, indicating that the data were reliable (Supplementary Fig. SF2).

Discussion

The study aimed at defining an alternative, well tolerated and easy to administer first-line therapy in patients with advanced EGC. Although biweekly pralatrexate at 100 mg/m^2 in combination with biweekly oxaliplatin was well tolerated, the study did not reach its primary end point of efficacy defined by radiographic response. On analyzing the adverse events only two DLTs were seen in the entire cohort and other adverse events were manageable with supportive therapy. Efficacy analysis showed a modest PFS and OS with this combination. The majority of patients had an ECOG performance status $\geq$1 reflective of the overall sick patient population. This pattern applies to general practice in the community where majority of patients are symptomatic and have concurrent nutritional compromise resulting in significant decline in performance status. This may have been a contributing factor to poor clinical outcome in this cohort in the setting of a reasonably well tolerated regimen.

We also evaluated the prognostic role that polymorphisms in folate pathway transport and metabolism genes play in pralatrexate treatment. Studies involving other anti-folates therapies, such as pemetrexed, have shown clinical outcome correlations for folate pathway gene variants (23). However, no such data have been reported in relation to pralatrexate. In the current study, there was variability in clinical outcomes for SNPs in *DHFR, GGH*, and *SLC19A1* specifically for associations with PFS and OS. The *SLC19A1* rs2838957 polymorphism was associated with an improved PFS and rs1051266 with improved OS. *SLC19A1* rs1051266 polymorphism is in linkage disequilibrium with several other *SLC19A1* polymorphisms, including

Table 4. Summary of genotype and outcomes.

Category	Gene	SNP rs id/ nucleotide	Genotype (*N*)	Number of events (% between allele groups)	Median survival, months (95% CI)	*P*[a]
Progression-free survival	***DHFR***	rs11951910 T>C	TT (25)	25 (71.4)	5.9 (3.6–6.6)	**<0.01**
			TC (10)	10 (28.6)	2.9 (1.8–3.8)	
			GG (13)	13 (35.3)	3.7 (1.9–5.5)	
	SLC19A1	rs2838957 G>A	GA (14)	14 (41.2)	5.6 (2.3–8.1)	0.06
			AA (8)	8 (23.5)	5.1 (1.8–9.7)	
			GA+AA (22)	**22 (62.9)**	**5.6 (3.0–7.4)**	**0.02**
	GGH	rs3780130 A>T	AA (18)	18 (51.4)	6.8 (5.4–7.2)	
			AT+TT (17)	17 (48.6)	11.4 (4.7–19.4)	**0.01**
		rs7010484 T>C	TT (16)	16 (45.7)	9.2 (6.4–19.4)	**0.04**
			TC+CC (19)	19 (54.3)	6.9 (4.7–10.4)	
Overall survival	***SLC19A1***	rs1051266 G>A	GG (11)	11 (31.4)	6.7 (2.3–7.4)	
			GA (16)	16 (45.7)	8.7 (5.7–22.2)	**0.03**
			AA (8)	8 (22.9)	11.1 (2.6–12.9)	
			GA+AA (24)	**24 (68.6)**	**10.1 (6.4–12.9)**	**0.01**

Note: Content in bold is statistically significant.
Abbreviation: *N*, number of samples with genotype.
[a]Log rank *P* values.

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rs2838958 that was previously associated with improved OS in a pemetrexed-based study (23). The *GGH* rs3780130 polymorphism was previously associated with reduced risk of grade 3 fatigue (23) and in this study was associated with improved OS. Furthermore, trends toward associations with OS and PFS for polymorphisms in *ATIC* (rs2030774), *GGH* (rs16930092), *GART* (rs6517177), *SLC19A1* (rs1023159, rs12659, rs3788189), and *TYMS* (rs699517) were also observed ($P = 0.06–0.09$), suggesting that some pralatrexate pathway genes may be contributing more to the variations in clinical outcomes.

We also attempted to elucidate predictive and prognostic biomarkers for this combination in advanced EGC. MicroRNAs are small non-coding RNAs that were first described in the nematode C. elegans, and are now known to regulate the expression of protein coding genes in various organisms. Over 800 microRNAs have been identified in humans. While changes in expression patterns of messenger RNA (mRNA) have been shown to be promising in chemosensitivity prediction in several human cancers, there are significant problems in their utilization in everyday clinical use. On the other hand, microRNAs are stable to degradation over long periods of time (30). This enables the use of RNA extracted from paraffin-embedded tissue, thereby enhancing and simplifying clinical utility. Therefore, a microRNA signature is potentially more feasible for general clinical use. Limited data exist for utilization of microRNA signatures for chemosensitivity prediction. Hence, we aimed to explore the role of microRNA signature as a predictive biomarker.

The microRNA profile of tumor epithelium in this cohort did not correlate with response or survival and hence could not be validated as a predictive biomarker. We obtained tissue from 29 of the 35 patients enrolled in the study. In 4 of these 29 patients the amount of RNA extracted was less than 3 μg emphasizing the challenges involved in tissue collection and acquisition in clinical trials. The sensitivity and specificity of biomarker analyses was limited in this small sample.

The association of pharmacogenomics and drug metabolism with efficacy and toxicity of an agent is well-recognized phenomenon. In this study, we used a novel combination of cytotoxic agents not previously used or systematically evaluated with respect to predictors of toxicity and efficacy. Modest activity of this regimen was demonstrated in EGC, with suggestion of pharmacogenomic factors influencing the efficacy of pralatrexate. Hence, it would be prudent to include pharmacogenomic analysis in related investigations of drugs targeting folate metabolic pathways. Ultimately, these data may prove to be valuable tools for individualization of therapy.

In conclusion, the combination of oxaliplatin and pralatrexate did not meet the primary end point of improved ORR in advanced EGC in the current study. SNP analysis revealed associations of variants in *DHFR*, *GGH*, and *SLC19A1* with clinical outcome. Therefore, pharmacogenomic analysis may be relevant to the clinical use of pralatrexate in other malignancies.

Disclosure of Potential Conflicts of Interest

U. Malhotra is a clinical director at Merck & Co., Inc. C. Fountzilas is a consultant/advisory board relationship member for AstraZeneca. P. Boland is an advisory board member for Boston Biomedical, Ipsen, Merck, Boerhinger Ingelheim, and Taiho. M. Opyrchal is an advisory board member for Novartis, AstraZeneca, and Pfizer, and reports receiving a commercial research grant from Pfizer and Beyer. N.I. Khushalani is an advisory board member for Bristol-Myers Squibb, EMD Serono, Merck, HUYA Bioscience, Regeneron, Genentech, Immunocore, Array, and Sanofi, received an honorarium from AstraZeneca, Data Safety Monitoring Board, reports receiving a Commercial Research Grant from Bristol-Myers Squibb, Merck, Celgene, Novartis, GlaxoSmithKline, HUYA Bioscience, Amgen, and Regeneron, and has ownership interest (including patents) in Bellicum Pharmaceuticals, Mazor Robotics, Amarin, and Transenterix. No potential conflicts of interest were disclosed by the other authors.

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Pralatrexate in Combination with Oxaliplatin in Advanced Esophagogastric Cancer: A Phase II Trial with Predictive Molecular Correlates

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