Evaluation of KRAS Mutations, Angiogenic Biomarkers, and DCE-MRI in Patients with Advanced Non–Small-Cell Lung Cancer Receiving Sorafenib

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Abstract

Purpose: Sorafenib, a multikinase inhibitor targeting Raf and VEGFR, has shown activity in unselected patients with non–small-cell lung cancer (NSCLC). At present there are no validated biomarkers indicative of sorafenib activity.

Experimental Design: Patients received sorafenib 400 mg BID daily to determine activity and tolerability and to measure its biological effects. KRAS mutation status (N = 34), angiogenesis markers (VEGF, bFGF, FLT-1, PLGF-1) and imaging with DCE-MRI (dynamic contrast enhanced MRI) to determine early changes in tumor vascular characteristics were evaluated. Three parameters \(K_{\text{trans}}, K_{\text{ep}},\) and \(V_e\) were measured by DCE-MRI at baseline and day 14 of cycle 1. Cytokine analysis was done on days 0, 14, 28, and 54.

Results: Thirty-seven patients with previously treated stage IV NSCLC were enrolled in this single-center phase II trial. In 34 evaluable patients, 2 had partial responses and 20 had stable disease for 3 to 17 months, a disease control rate of 65%. The median progression-free survival (PFS) was 3.4 months, and median overall survival (OS) was 11.6 months. Toxicity was consistent with the known side effects of sorafenib. KRAS (32%) and EGFR mutations (22%) showed no correlation with response, PFS, or OS. \(K_{\text{ep}}\) was significant in predicting an improvement in OS \((P = 0.035)\) and PFS \((P = 0.029)\). Cytokine analysis demonstrated an improved OS for bFGF day 0 \((<6\) vs. \(>6\) pg/mL; \(P = 0.042)\), whereas a PFS benefit was seen with bFGF at day 28 \((<6\) vs. \(>6\); \(P = 0.028)\).

Conclusions: KRAS and EGFR mutational status showed no correlation with response, PFS, or OS. Radiologic and cytokine changes may act as biomarkers indicative of early angiogenesis inhibition.

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Introduction

Treatment outcomes for advanced non-small-cell lung cancer (NSCLC) have been limited by the empiric administration of cytotoxic chemotherapy (1). Small-molecule tyrosine kinase inhibitors (TKI) have demonstrated single-agent activity in a wide variety of solid tumors including NSCLCs (2); however, the lack of validated predictive factors for many of these targeted treatments remains problematic. Recent evidence has highlighted the importance of individualizing therapy based on certain molecular characteristics (EGFR mutations and EML4-ALK translocations; refs. 3–5). KRAS mutations are present in approximately 30% of NSCLCs and are responsible for the proliferation signaling of the RAS/RAF/MEK (kinase)/ERK (extracellular signal regulated kinase) pathway and indicate a poor prognosis and poor response to EGFR inhibitors (6, 7). Therapeutic targeting of the Ras pathway has so far been unsuccessful. RAF serine–threonine kinases are the principal effectors of RAS and are considered an important target for cancer therapy. Sorafenib is a multikinase inhibitor that inhibits C-RAF and B-RAF; VEGFRs 1, 2, and 3, and PDGF \(\beta\) (platelet derived growth factor \(\beta\)); Flt3; RET; and c-KIT (8–11). Sorafenib has shown activity in preclinical models of NSCLC and several phase I (9, 12–15) and phase II trials (16, 17). To date there are limited data with regard to sorafenib sensitivity among NSCLC patients with different KRAS mutational status.

VEGF is upregulated in many tumors (18). Unfortunately, there are no validated biomarkers in clinical practice.
Sorafenib in Patients with KRAS Mutant/Wild-Type NSCLC

Translational Relevance

KRAS mutations are present in approximately 30% of non–small-cell lung cancers (NSCLC) and indicate a poor prognosis and a poor response to EGFR inhibitors. Currently, no direct RAS inhibitor has proven clinically effective, and agents such as sorafenib that bypass RAS and inhibit effector molecules downstream of the mutated GTPase (e.g., RAF) are being evaluated. To our knowledge, this article is the first completed study of the direct impact of RAS mutations in NSCLC patients treated with sorafenib. The most striking observation from this report is that sorafenib inhibited the growth of NSCLC in a manner independent of KRAS mutational status, with no differences in response rate, progression-free survival, or overall survival being detected between patients with KRAS wild-type or mutant tumors. The inhibitory effect of sorafenib in KRAS wild-type tumors was also independent of whether there was a mutant EGFR gene present or not.

Originally, patients with squamous cell carcinoma were allowed on study but a second protocol amendment excluded these patients due to side effects seen in the ESCAPE trial (24). The study followed the current guidelines of the International Conference on Harmonization for good clinical practice and the Declaration of Helsinki.

Study design

The primary objective of this single-arm study was to determine the response rate (RR) and toxicity of sorafenib administered at a dose of 400 po mg BID daily in a 28-day cycle in relapsed or recurrent NSCLC patients. Secondary objectives included a correlation of the patient’s response to treatment with KRAS mutation status and with changes in angiogenic cytokines, an evaluation of the application of DCE-MRI (dynamic contrast enhanced MRI) to determine early changes in tumor vascularity during treatment, and an evaluation of time to progression and overall survival (OS). Treatment continued until objective evidence of tumor progression or intolerable drug-related side effects. Adverse events were defined by the NCI Common Terminology Criteria for Adverse Events (NCI CTCAE) version 3.0. Two dose reductions were allowed for clinically significant toxicities attributed to sorafenib while on study: dose level 1 was 200 mg BID and dose level 2 was 200 mg OD. Doses were not reescalated once toxicities had recovered. Drug could be held a maximum of 3 weeks before discontinuation from study.

Imaging studies: DCE-MRI

DCE-MRI was used to evaluate changes in vascularity at baseline and at day 14 (+/−3 days) of cycle 1. DCE-MRI was done with a 1.5-T MR system (Philips Achieva) using a dedicated receive-only 6-channel phased array coil (see Supplementary text). Data from DCE-MRI were analyzed using a 2-compartment (Kety) model, also known as the general kinetic model (GKM; refs. 25, 26). The GKM model analysis was done with an IDL-based (Interactive Data Language; Research Systems Inc.) research tool (Cine Tool; GE Healthcare). Manual region-of-interest measurements were obtained from each slice of the target lesion. The GKM model produces 3 parameters: \( K_{ep} \), the reverse contrast transfer rate; \( K_{trans} \), the forward contrast transfer rate; and \( V_e \) the extravascular fraction. Baseline and follow-up after treatment cycles \( K_{ep} \), \( K_{trans} \), and \( V_e \) values were obtained.

Cytokine analysis

Serial plasma samples were collected from all patients in an EDTA-containing vacutainer at pretreatment (baseline – day 0), and on days 14, 28, and 54. After centrifugation, the samples were aliquoted, immediately frozen, and stored at −80°C. Plasma analysis included evaluation of VEGF, placental-derived growth factor (PLGF), basic fibroblast growth factor (bFGF), and VEGF receptor 1 (sVEGFR1 or FLT-1), using multiplex array plates from Meso-Scale Discovery. The concentrations of the cytokines were determined with recombinant standards and expressed as picograms per milliliter.
KRAS/EGFR/BRAF mutation identification

Five to ten 5-μm sections from formalin-fixed, paraffin-embedded (FFPE) tissue sections were deparaffinized by standard methods. Macrodissection was done on selected specimens to obtain at least 10% tumor cell content for DNA isolation, as necessary. DNA isolation was carried out with the QIAmp FFPE DNA Kit on an automated QIAcube instrument (Qiagen). Targeted analysis of KRAS codons 12, 13, and 61 was done using pyrosequencing technology on a PyroMark Q24 instrument (Qiagen) and the PyroMark Q24 KRAS v2.0 kit (Qiagen), as described by Ogino and colleagues (27). Targeted analysis for EGFR mutations involving exon 20 codon 790, exon 21 codons 858/861/863 was done using pyrosequencing while exon 19 deletions were assessed by capillary electrophoresis using a Genetic Analyzer 3130xl (Applied Biosystems), as described by Pan and colleagues (28). Primers for the EGFR pyrosequencing reactions were designed in our laboratory and are available on request. For BRAF V600 mutation detection, the extracted DNA was subjected to an initial PCR using a single primer set (sequence available on request) encompassing codon V600. Pyrosequencing was carried out on a Qiagen PyroMark Q24 system.

Statistical considerations

The study was conducted using a phase II optimal design in order to determine if sorafenib was able to be associated with an RR [(PR (partial response) + CR (complete response)] that could rule out 5% (P₀ = 0.05) in favor of a more desirable 20% RR (P₁ = 0.20). Using α = 0.10 (probability of accepting a poor drug) and β = 0.10 (probability of rejecting a good drug), initially 12 patients were to be enrolled onto the trial. If none of the 12 patients responded, then accrual would end; if 1 or more of the first 12 patients had a CR or a PR, then accrual would continue until a total of 37 patients with measurable disease had been enrolled. If 4 or more of 37 had a response, then this would warrant further investigation in a subsequent trial. The associations between KRAS and EGFR mutations and response [PR + SD vs. PD (progressive disease)] were determined by a Fisher’s exact test. Comparisons of continuous parameters were made using an exact Wilcoxon rank-sum test. The difference from baseline to days 14, 28, and 54 was determined by a Wilcoxon signed rank test. Survival and progression-free survival (PFS) analyses were initially done using univariate Cox models to screen for the association between outcome and values of a continuous parameter less than or greater than the median value. For the cases in which a trend was identified, the P value was confirmed by a log-rank test P value. In view of the exploratory nature of these analyses, any P values reported have not been adjusted for multiple comparisons, and the results of these analyses are considered hypothesis generating.

Results

Patient characteristics

Baseline demographic data and disease characteristics are listed in Table 1. All patients had received prior therapy, with 21 patients (57%) having had at least 2 prior regimens. Seventeen patients (46%) had received only 1 previous regimen prior to study enrollment. In total, 43% had received prior EGFR TKIs and 40% had received prior bevacizumab.

Efficacy

All patients had PD at the time of enrollment. The median duration of treatment was approximately 3 months.
(97 days, range, 12–517 days). Three patients were considered not evaluable for response, 1 patient withdrew from study after 4 weeks, and the 2 others withdrew because of a lack of target lesions. In total, 2 partial responses (PR; 6%) were seen (Fig. 1). The first patient had metastatic squamous cell carcinoma and had received only 1 previous regimen of carboplatin and paclitaxel for 6 cycles. His tumor was wild type for KRAS, EGFR, and BRAF. The second patient had metastatic adenocarcinoma to the brain and adrenal gland. He had received 1 previous chemotherapy regimen of carboplatin, gemcitabine, and bevacizumab before commencing sorafenib. His tumor was wild type for KRAS and BRAF and not enough tissue was available for EGFR analysis.

Median PFS for evaluable patients was 3.4 months and median OS was 11.6 months (Fig. 2). The disease control rate (DCR) was 65% with 2 PRs (6%) and 20 SDs (59%). In patients who had received only 1 previous therapy, 2 PRs and 7 SDs were seen. In an evaluation of all 37 patients, SD greater than 3 months (3 cycles; range from 3 to 17 months) was documented in 20 patients (54%), with a median duration of 5.4 months. One patient had clinical disease progression and died 3 weeks after sorafenib was discontinued. There were no differences in terms of RR, PFS, or OS depending on whether patients had 1 or multiple lines of prior therapy or between squamous cell and adenocarcinomas.

**KRAS and EGFR mutations**

Table 2 summarizes the characteristics of patients with either wild type or mutant KRAS/EGFR. Three of 37 patients did not have tissue available for KRAS analysis. KRAS mutations occurred in 11 of 34 patients (32%). Fourteen patients (38%) did not have evaluable tissue for EGFR mutation analysis. Of the remaining 23 patients, there were 5 (22%) subjects with an activating EGFR mutation. There was reciprocal exclusion of EGFR and KRAS mutations, as reported by others (29). Interestingly, 3 (8%) never smoking patients with adenocarcinomas demonstrated KRAS mutations. There was no correlation between the mutational status of KRAS or EGFR and RR, PFS, or OS. The DCR observed in KRAS mutant and KRAS wild-type patients was 60% and 71%, respectively ($P = 0.69$). The DCR observed in EGFR mutant and EGFR wild-type patients was 40% and 69%, respectively ($P = 0.33$).

**Plasma biomarker analysis**

All 37 patients were evaluable for analysis. Thirty-two patients (86%) had plasma drawn at baseline, whereas only 14 samples (38%) were available for day 54 analysis. Increases in VEGF and decreases in VEGR1 from baseline to day 54 were detected. Increases in plasma PLGF levels were also seen at these time points (Supplementary Table 1). The 4 cytokines were each divided at their respective median values and evaluated for their association with OS and PFS. Four of the parameters evaluated demonstrated an association with improved OS and PFS by having univariate 2-tailed $P < 0.10$ values (Supplementary Tables 2 and 3). Of these parameters, bFGF at baseline <6 versus >6 was significant for OS ($P = 0.042$), and similar levels on day 28 were significant for PFS ($P = 0.028$; Fig. 3). The difference between day 28 and day 0 PLGF was significant for OS (<11 vs. >12; $P = 0.0027$; Supplementary Fig. 2). Cytokine analysis did not predict response to sorafenib.

**DCE-MRI**

Twenty-six patients (70%) had DCE-MRI scans at baseline and on day 14 (±3 days) of cycle 1. Target lesions were localized in the lung (20 patients), adrenal gland (2 patients), liver (1 patient), mediastinum (1 patient), chest...
wall (1 patient), and neck (1 patient). Decreases in either $K_{ep}$ or $K^{trans}$ were seen in 21 patients (81%), whereas an increase was observed in 5 patients (19%; Supplementary Fig. 1). $K_{ep}$, $K^{trans}$, and $V_e$ measurements at day 0, day 14, and the difference between the day 14 and the day 0 measurements (day 14 – day 0) were each divided at their respective median values and evaluated for their association with RR, PFS, and OS. In a univariate exploration the $K_{ep}$ difference of less than –0.15 versus greater than –0.14 was statistically significant, with an improved OS ($P = 0.035$) and PFS ($P = 0.029$) for those patients demonstrating a $K_{ep}$ difference of less than –0.15 compared with patients with a $K_{ep}$ difference of greater than –0.14 (Fig. 3). Nonsignificant differences were associated with $K^{trans}$ and $V_e$. DCE-MRI did not help predict response to sorafenib.

Safety
The toxicities of sorafenib have been well documented, and side effects similar to those previously described were seen in this study (Table 3). In total, 16 patients tolerated the full dose of 400 mg po BID, but 21 patients needed a dose reduction (57%). Of these, 11 patients required 1 dose reduction and 10 required a second reduction to 200 mg po OD. All patients who were reduced to dose level 2 tolerated sorafenib at this dosage. Twelve patients (32%) required a dose reduction during cycle 1. The most frequent grade 3 drug-related adverse events were hypertension (16%), hand/foot syndrome (14%), dyspnea (14%), and hypophosphatemia (14%). One patient developed a squamous cell carcinoma of the skin while on study, which was treated by excision and discontinuation of drug.
A second patient developed a keratoacanthoma, which was removed and observed while continuing on drug. The most common reason for treatment discontinuation was disease progression ($n = 33; 94\%$). To date 26 patients have died; 7 (27\%) died within 60 days from coming off study. There was 1 death while on study secondary to clinical progression.

**Discussion**

In this trial, sorafenib demonstrated activity with an RR of 6\%, a DCR of 65\%, a PFS of 3.4 months, and an OS of 11.6 months. Two other phase II clinical trials have reported activity of sorafenib in heavily pretreated patients with NSCLC, with an OS of 6.7 months, median PFS of 2.7 months, and SD in 59\% (16), and in ECOG 2501 a DCR of 47\% and a median PFS of 3.6 months (17). Little is known of the possible differences in the sensitivity of NSCLC to sorafenib according to KRAS mutational status. Furthermore, the identification of biomarkers predictive of treatment outcomes for anti-VEGF therapies has so far met with limited success. In the BATTLE trial, the overall DCR for patients treated with sorafenib was 58\%. Subset analyses showed DCR of 61\% in patients with KRAS mutations versus 56\% KRAS wild type but only a 23\% DCR in patients with an EGFR mutation compared with 64\% in patients...

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Abbreviations: Adeno-BAC, adenocarcinoma with bronchioloaveolar features; WT, wild type; N/A, not available.
Without an EGFR mutation (P = 0.048; ref. 30). Here we report a DCR of 60% in patients with KRAS mutations versus 71% in KRAS wild type (P = 0.69) and a DCR of 40% in EGFR mutations versus 69% in patients without an EGFR mutation (P = 0.33). Smit and colleagues observed 3 PRs and a median PFS of 3 months (95% CI: 2.2–3.8 months) in 10 patients with previously treated advanced NSCLC all harboring KRAS mutations (31).

Currently, no direct RAS inhibitor has proven clinically effective and agents such as sorafenib that bypass RAS and inhibit effector molecules downstream of the mutant GTPase (e.g., RAF) are being evaluated. Preclinical data have suggested that sorafenib inhibits cell growth by inducing $G_1$ arrest in NSCLC cell lines independent of KRAS genotype (32). Here we report that sorafenib inhibited the growth of NSCLC in a manner independent of KRAS mutational status, with no differences in RR, PFS, or OS being detected between patients with KRAS wild-type or mutant tumors. The inhibitory effect of sorafenib in KRAS wild-type tumors was also independent of whether there was a mutant EGFR gene present or not. Preclinical studies have indicated that sorafenib blocks the ERK signaling pathway only in wild-type KRAS tumors and inhibits NSCLC cell growth by targeting B-RAF in cells with wild-type KRAS and C-RAF in cells with mutant KRAS (32, 33). These results need to be validated in clinical trials and were not assessed in this study.

Figure 3. Cytokine and DCE-MRI analysis. Exploratory analysis demonstrating potential trends toward an association with OS and PFS by having univariate 2-tailed P < 0.10 values included bFGF and the reverse constant transfer rate ($K_{ep}$) as measured via DCE-MRI. A, patients with baseline (day 0) bFGF levels <6 pg/mL had an improved OS compared with patients with higher baseline bFGF levels >6 pg/mL. B, Patients with lower bFGF levels <6 pg/mL as measured post-cycle 1 (day 28) had an improved PFS than patients with higher day 28 bFGF levels >6 pg/mL, indicating a potential prognostic and predictive role for bFGF. Similarly, $K_{ep}$ as measured by DCE-MRI may act as a radiologic biomarker. Patients demonstrating a $K_{ep}$ difference of less than −0.15 (the difference between the day 14 and the day 0 measurements, i.e., day 14−day 0) compared with patients with a $K_{ep}$ difference of greater than −0.14 showed (C) an OS benefit and (D) a PFS benefit.
for each class of drug it may be possible to identify certain cytokine changes that occur during treatment that may serve as pharmacodynamic or efficacy markers. In this trial, we noted an increase in baseline VEGF levels and a decrease in sVEGFR-1, which have been reported in other studies and likely represent a class effect. The predictive role of pretreatment VEGF levels in patients with NSCLC and in patients treated with anti-angiogenic therapy remains controversial, with some suggestion that bioavailable rather than circulating VEGF may provide the most predictive value (34–37). Germ line polymorphisms in VEGF are also being evaluated as a means to help predict patients likely to respond to sorafenib (38). In an exploratory analysis of 4 plasma cytokines, we found distinct patterns of cytokine changes that may act as predictors of response to sorafenib. BFGF levels at day 0 and day 28 showed significance in terms of an OS ($P = 0.042$) and PFS benefit ($P = 0.028$) and may act as prognostic and predictive biomarkers (Fig. 3). These correlative studies are considered hypothesis generating and may form the basis for future trials.

Functional and molecular imaging may also be used for pretherapy molecular phenotyping and may prove effective as pharmacodynamic predictive biomarkers. DCE-MRI is noninvasive and is sensitive to tumor perfusion parameters such as vascular volume, vascular permeability, and flow. Typically, aggressive tumors are characterized by a rapid enhancement followed by a subsequent rapid washout period (26, 39). In this study, $K_{ep}$ showed a significant predictive value for OS ($P = 0.035$) and PFS ($P = 0.029$). $K_{ep}$ is considered more robust than the other parameters since it is not dependent on the T1 values of the tissue or $V_c$ (26). Although $K_{trans}$ and $K_{ep}$ are correlated with each other, it is not surprising that one parameter might better predict OS and PFS than another. For instance, $K_{trans}$ can be influenced by changes in $V_c$ values after treatment, variability in image noise, and T1 measurement and is very sensitive to patient motion. In this study, most of the target lesions were localized to the lungs, adrenal glands, and liver, all of which are subject to significant motion.

In conclusion, this trial demonstrates that sorafenib provides clinical benefit for patients with heavily pretreated advanced NSCLC irrespective of their KRAS mutational status. Although this study is limited by the relatively small sample size and varied population, it is indicative of the population most often seen in thoracic oncology clinics whereby a wide array of histologies and prior therapies are encountered. Establishing predictive biomarkers for anti-angiogenics remains a significant challenge as discovery and validation will have to be tailored to the known mechanisms of action of a certain agent in a certain disease and will require standardization of biomarker assays amongst protocols. Challenges to overcome include establishing adequate criteria to measure response and the need for spatially resolved dynamic biomarkers to meet the heterogeneous and dynamic nature of cancer. Preliminary biomarker data are emerging but mostly from single-arm trials, making it difficult to ascertain whether the marker is prognostic or predictive. A combination of vascular permeability imaging and circulating factors measured at various time points may yield a “composite biomarker” to make robust predictions. Ultimately, these data will have to be tested and validated in large, well-designed, prospective clinical trials.

**Disclosure of Potential Conflicts of Interest**

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