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Increased *EGFR* Gene Copy Number Detected by Fluorescent In Situ Hybridization Predicts Outcome in Non–Small-Cell Lung Cancer Patients Treated With Cetuximab and Chemotherapy

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Abstract

Purpose—Epidermal growth factor receptor (*EGFR*) gene copy number detected by fluorescent in situ hybridization (FISH) has proven to be useful for selection of non–small-cell lung cancer (NSCLC) patients for treatment with EGFR tyrosine kinase inhibitors. Here, we evaluate *EGFR* FISH as a predictive marker in NSCLC patients receiving the EGFR monoclonal antibody inhibitor cetuximab plus chemotherapy.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Patients and Methods—Two hundred twenty-nine chemotherapy-naive patients with advanced-stage NSCLC were enrolled onto a phase II selection trial evaluating sequential or concurrent chemotherapy (paclitaxel plus carboplatin) with cetuximab.

Results—*EGFR* FISH was assessable in 76 patients with available tumor tissue and classified as positive (four or more gene copies per cell in ≥ 40% of the cells or gene amplification) in 59.2%. Response (complete response/partial response) was numerically higher in FISH-positive (45%) versus FISH-negative (26%) patients ($P = .14$), whereas disease control rate (complete response/ partial response plus stable disease) was statistically superior (81% *v* 55%, respectively; $P = .02$). Patients with FISH-positive tumors had a median progression-free survival time of 6 months compared with 3 months for FISH-negative patients ($P = .0008$). Median survival time was 15 months for the FISH-positive group compared with 7 months for patients who were FISH negative. ($P = .04$). Furthermore, survival favored FISH-positive patients receiving concurrent therapy.

Conclusion—These results are the first to suggest that *EGFR* FISH is a predictive factor for selection of NSCLC patients for cetuximab plus chemotherapy. Prospective validation of these findings is warranted.

INTRODUCTION

Lung cancer is the most frequent cause of cancer death.[1] The overall prognosis remains poor, with 15% of patients surviving 5 years.[2] Gefitinib and erlotinib, which are tyrosine kinase inhibitors (TKIs) that target the epidermal growth factor receptor (EGFR), provide objective response in 8% to 15% of patients with advanced non–small-cell lung cancer (NSCLC) progressing after initial chemotherapy. In a randomized, placebo-controlled, phase III study, erlotinib improved survival in NSCLC patients previously treated with chemotherapy.[3] Despite independent activity of chemotherapy and EGFR TKIs in NSCLC, the addition of gefitinib or erlotinib to chemotherapy failed to improve survival in four large randomized trials when compared with chemotherapy alone.[4-7] After these results, interest in identifying predictive biomarkers to EGFR TKIs intensified. EGFR pathway analysis of NSCLC cell lines and patient tumor tissue has described predictive value for several potential biomarkers of EGFR TKI activity, including EGFR protein expression by immunohistochemistry, *EGFR* gene copy number by fluorescent in situ hybridization (FISH) or chromogenic in situ hybridization, *EGFR* activating mutations, as well as *KRAS* mutations, p-MAPK, and p-AKT.[8] *EGFR* mutations, which typically predict rapid objective response to EGFR TKIs, are most frequent in never-smokers and more common in those patients with clinical characteristics associated with response (ie, patients with adenocarcinomas, Asian race, and female sex). Certain *EGFR* mutations, such as deletions in exon 19, associate with high response rate and prolonged survival after EGFR TKIs, whereas point mutations in exon 20 (T790) associate with acquired resistance.[8] High *EGFR* gene copy number has been consistently associated with a favorable outcome after EGFR TKI therapy, whereas *KRAS* mutations are consistently associated with a poor outcome.[9-14]

The role of the EGFR-targeting monoclonal antibodies in the therapy of NSCLC has not yet been clarified. Cetuximab (Erbitux; Bristol-Myers Squibb Co, New York, NY/ImClone Systems Inc, New York, NY), a chimerized antibody of the immunoglobulin G1 subclass, has proven efficacy in colorectal cancer[15] and head and neck cancer.[16,17] In NSCLC, a phase II study in pretreated advanced-stage patients showed a response rate of 4.5%, but disease control rates (DCRs) and overall survival were comparable to that achieved with pemetrexed, docetaxel, and erlotinib in similar groups of patients.[18] Early phase II trials and randomized phase II trials of cetuximab plus concurrent chemotherapy versus chemotherapy alone in unselected chemotherapy-naive advanced NSCLC patients favored the combination.[19-21] In view of previous phase III studies showing no benefit of concurrent

EGFR TKI plus chemotherapy combinations over chemotherapy alone, the Southwest Oncology Group (SWOG) sought to study cetuximab in a randomized phase II selection design, comparing sequential versus concurrent cetuximab and paclitaxel-carboplatin chemotherapy (S0342), to select the most appropriate regimen to test against chemotherapy alone in the phase III setting. Preliminary clinical data from S0342 demonstrated comparable response rates and progression-free survival (PFS) and overall survival data in both arms of the study.[22] Here, for the first time to our knowledge, we report that *EGFR* gene copy number detected by FISH predicts outcomes in patients with advanced-stage NSCLC receiving these cetuximab plus chemotherapy combinations.

PATIENTS AND METHODS

Patients with advanced NSCLC not previously treated with chemotherapy or radiotherapy were randomly assigned either to receive paclitaxel 225 mg/m^2 and carboplatin (area under the curve = 6) every 3 weeks plus concurrent cetuximab 400 mg/m^2 by 2-hour infusion on day 1 in week 1 and, thereafter, 250 mg/m^2 by 1-hour infusion weekly for four cycles followed by maintenance cetuximab or to receive sequential paclitaxel plus carboplatin for four cycles followed by cetuximab (Fig 1). Treatment was continued until progressive disease or unacceptable toxicity. After progressive disease, patients were treated at investigator discretion. No data regarding second-line therapy were collected. Eligible patients were required to have stage IIIB (pleural infusion) or stage IV disease without brain metastases, a performance status of 0 to 1, and adequate organ function. The primary end point was survival. All patients had signed informed consent.

EGFR *Gene Copy Number Analysis by FISH*

Available tissue samples were submitted to the SWOG tumor bank at the University of Colorado Cancer Center and evaluated for tumor content. Formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin, and a quality control assessment of the tumor tissue was made before FISH analysis was performed to ensure that sufficient material was available.

FISH analysis was performed as previously described.[9,10] Tumors with four or more copies of the *EGFR* gene in ≥ 40% of the cells (high polysomy) or tumors with *EGFR* gene amplification (gene-to-chromosome ratio ≥ 2 or presence of gene cluster or ≥ 15 gene copies in ≥ 10% of the cells) were considered to be FISH positive, where as all other tumors were considered to be FISH negative. Two observers independently scored 50 tumor cells each in at least four tumor areas. In case of discordance, a third observer performed the analysis, and the majority score was assigned to the specimen. All FISH analyses were performed in a blinded fashion without access to the patient clinical characteristics or treatment outcome. FISH analyses were performed only on histologic material.

Statistical Design

The statistical analysis was conducted on an intent-to-treat basis, and all 76 patients with FISH data are included. Overall survival time was measured from the date of trial entry until death and was estimated using the Kaplan-Meier method. PFS was measured from trial entry until documented disease progression (by Response Evaluation Criteria in Solid Tumors) or death. Response was assessed for the entire course of treatment using Response Evaluation Criteria in Solid Tumors, and tests of association between response and FISH status were performed using Fisher's exact test. Multivariate analyses and univariate hazard ratios (HRs) for comparisons of FISH-positive versus FISH-negative groups were generated by Cox proportional hazards regression analysis using the SAS System for Windows Version 9.0 PHREG procedure (SAS Institute, Cary, NC).

RESULTS

Two hundred twenty-nine patients were enrolled onto the S0342 clinical trial. Seventy-six patients gave informed consent for correlative science participation (S9925 master correlative science protocol) and had assessable tumor tissue. Patient characteristics were similar between the overall study group and patients with *EGFR* FISH analysis performed (Table 1). Of the 76 patients in whom *EGFR* FISH testing was performed, 46 patients (61%) had adenocarcinoma, 17 patients (22%) had squamous cell carcinoma, and the rest of patients had NSCLC not otherwise specified or other NSCLC histologies. Ten patients (13%) had stage IIIB disease, and 66 patients (87%) had stage IV disease. Forty patients were treated in the concurrent arm, and 36 patients were treated in the sequential arm. Smoking status assessment showed 83% ever-smokers (current and former, 67% of females and 97.5% of males, $P = .0004$); 17% of patients were never-smokers (Table 1).

A total of 84 specimens from 76 S0342 patients were investigated for FISH analysis. Discrepancies between the first and second readers were found in nine specimens (10.7%), which is within the expected range of discrepancies for the laboratory (5% to 15%).

Increased *EGFR* gene copy number (FISH positive) was present in 45 patient specimens (59.2%), whereas 31 specimens (40.7%) were FISH negative. Twenty-five (62.5%) of 40 patients were FISH positive in the concurrent treatment arm, and 20 (55.5%) of 36 patients in the sequential treatment arm were FISH positive. Treatment results for all 229 patients enrolled onto the S0342 trial were similar between the concurrent and sequential arms, with no difference in overall response rate (34% *v* 31%, respectively), stable disease rate (34% *v* 39%, respectively), DCR (68% *v* 69%, respectively), median PFS (4 months in both arms), or median overall survival (11 *v* 10 months, respectively).[21] Outcomes were similar between the overall patient population and patients assessed for *EGFR* FISH. The median survival time was 10.5 months for all of the patients in the study (N = 209) and 11 months in patients assessed for *EGFR* FISH (n = 76; $P = .29$). There was a statistically insignificant trend for association of smoking status and *EGFR* FISH status ($P = .35$).

Study results according to FISH status and treatment arms are listed in Table 2. Among the S0342 patients assessed by *EGFR* FISH, the median survival time was 15 months compared with 7 months for patients with a positive versus negative test, respectively (HR = 0.58, $P = .046$; Fig 2). One-year survival rate was 58% for FISH-positive patients and 32% for FISH-negative patients. FISH-positive patients had a superior survival in both treatment arms, although the difference was only significant in the concurrent arm. In the concurrent arm (Fig 3), the median survival time was 16 months for FISH-positive patients compared with 8 months for FISH-negative patients (HR = 0.43, $P = .03$), whereas in the sequential treatment arm (Fig 4), the median survival time was 15 months for FISH-positive patients compared with 7 months for the FISH-negative patients (HR = 0.83, $P = .65$). The 1-year survival rate was 64% *v* 20% in the concurrent arm and 50% *v* 44% in the sequential arm for FISH-positive and FISH-negative patients, respectively.

Median PFS time was significantly longer (6 months) in the FISH-positive group compared with the FISH-negative group (3 months; HR = 0.45; $P = .0011$; Fig 2). A significant difference in PFS was seen between FISH-positive and FISH-negative patients in each of the treatment arms (concurrent arm: 5 *v* 3 months, respectively; HR= 0.45; $P = .02$; sequential arm: 6 *v* 3 months, respectively; HR = 0.46; $P = .03$; data not shown).

The overall response rate (complete plus partial response) was 37% (n=27) for the 73 response assessable patients in the *EGFR* FISH group, 36% in the concurrent arm, and 38% in the sequential arm. One patient in the sequential arm showed improvement from stable disease to partial response during the cetuximab maintenance treatment. (Two patients

improved from stable disease to partial response on the concurrent arm during cetuximab maintenance.) The DCR (objective response + stable disease) was 70% (51 of 73 patients; 69% in the concurrent arm and 71% in the sequential arm), which was not significantly different from the overall study population. Within the FISH-positive group (both treatment arms), the overall response rate was 45% (19 of 42 patients) compared with 26% in the FISH-negative group (eight of 31 patients), but the difference was not significant (P= .14). The DCR was significantly higher in FISH-positive patients (81%) than in FISH-negative patients (55%; P = .02). When analyzed within treatment arms, both response rate and DCR were numerically but not statistically higher in the FISH-positive versus FISH-negative subgroup (concurrent arm: response rate, 42% *v* 27%; DCR, 79% *v* 53%, respectively; sequential arm: response rate, 50% *v* 25%; DCR, 83% *v* 56%, respectively).

Altogether, 47 patients received poststudy therapy, including 17 of 31 patients in the FISH-negative group (four patients received EGFR TKI) and 30 of 45 patients in the FISH-positive group (six patients received EGFR TKI). There was no imbalance between the groups regarding poststudy therapy.

A multivariate Cox regression model including treatment arm and *EGFR* FISH status revealed a significant effect for *EGFR* FISH status in favor of FISH positivity (P = .049) even when adjusting for treatment arm. The effect of treatment arm was not significant (P = .42). A test for interaction between FISH and treatment arm was not significant (P = .25). The role of smoking status was analyzed in a multivariate Cox model. In a model that included FISH status, smoking status (current/former *v* never) did not represent a significant addition (P = .79).

DISCUSSION

S0342 is the first study to report that increased *EGFR* gene copy number by FISH predicts clinical outcomes after cetuximab-based therapy in patients with NSCLC. We previously reported that *EGFR* FISH portends a poor prognosis in NSCLC and is a reliable marker for prediction of clinical outcome after treatment with an EGFR TKI (ie, gefitinib),[9-11,23] and others have reported the predictive value of *EGFR* FISH for erlotinib.[12] Although these EGFR TKIs are well established in the treatment of NSCLC, to date, the biologic activity of cetuximab in this tumor type has remained poorly defined. Therefore, our results, although requiring prospective validation, are provocative because they are indicative of a clinical effect of this EGFR-targeting monoclonal antibody, which is associated with inhibition of the biologic target. Moreover, our results suggest that the addition of cetuximab to chemotherapy may reverse the underlying poor prognosis associated with *EGFR* FISH positivity, similar to the effects of trastuzumab plus chemotherapy in *HER-2* FISH-positive breast cancer patients.[24] In S0342, FISH-positive patients achieved a remarkable overall survival time (median, 15 months) when compared with the FISH-negative group (median, 7 months). In FISH-positive patients, the median PFS time was 6 months (*v* 3 months for the FISH-negative patients), and the objective response rate was 45% (*v* 26% for the FISH-negative patients). Furthermore, more than half of the patients in our study (59%) tested positive by FISH, increasing the potential clinical importance of our findings. Although this FISH-positive rate of 59% may seem somewhat high, it is not unexpected considering the underlying patient population within this cooperative group study (ie, primarily adenocarcinoma [61%] and almost one-half female [47%]). In another recent study from our group, the FISH-positive rate was 54%.[13]

Previously, several single-arm and randomized phase II trials combining cetuximab with chemotherapy in advanced NSCLC patients suggested higher response rates and longer survival times than might be expected for chemotherapy alone.[19-21] Subsequently, several

randomized phase III trials of chemotherapy alone versus chemotherapy plus cetuximab were initiated. The results of our study suggest that these all-comer trial designs may prove to be suboptimal for demonstrating the efficacy of cetuximab-chemotherapy combinations. Preliminary data from one of these phase III trials (study 099) reported that the primary end point of prolongation in disease-free survival was not met, although all end points favored the cetuximab-containing arm.[25] Unfortunately, the study had no prespecified biomarker analysis. Because our study indicates that *EGFR* FISH-positive patients derive considerable benefit from a chemotherapy-cetuximab combination, as defined by response rate, PFS, and overall survival, it is possible, but unproven, that benefit from cetuximab was diluted out by an undefined *EGFR* FISH-negative population. In support of this explanation are the preliminary results of a more recent phase III trial of chemotherapy with or without cetuximab (FLEX), in which patient eligibility required positive EGFR protein expression by immunohistochemistry. Using this approach, the primary end point of improved survival was achieved.[25a] We hypothesize that prospective trials using *EGFR* FISH analysis to select patients with NSCLC for cetuximab-based therapy will optimize clinical benefit from this agent. Testing this approach in other cancers is also appealing, including head and neck cancer, where positive results for cetuximab-based therapy are reported and FISH positivity is associated with poor outcome in the absence of EGFR-targeted therapy.[16,17,26,27]

Our results suggest that there may be inherent differences that distinguish cetuximab-chemotherapy combinations from EGFR TKI combination therapies. Four large randomized phase III trials comparing chemotherapy alone with chemotherapy plus an EGFR TKI showed no benefit for the combination.[4-7] Potential explanations for lack of benefit include nonselection of patients for a predictive biomarker and a negative interaction between concurrently administered chemotherapy and EGFR TKIs.[28] These results led SWOG to conduct the current randomized phase II trial evaluating cetuximab plus chemotherapy administered either concurrently or sequentially in a pick-the-winner design for subsequent phase III testing against chemotherapy alone. Outcomes were favorable in both arms, meeting prespecified criteria for further study.[22] A comparison of the concurrent versus sequential arms of S0342 in FISH-positive patients showed that, although similar results were achieved overall, survival data were significantly improved only in the concurrent arm. These results suggest considerable benefit for FISH-positive patients whether cetuximab is administered concurrently or sequentially, where as FISH-negative patients fare poorly with either mode of therapy. Whether similar results could be achieved by using FISH to select patients for EGFR TKI–chemotherapy combinations remains undetermined, but the recently reported results from TRIBUTE suggest otherwise.[29,30] In our analysis of *EGFR* FISH in this large phase III study of chemotherapy with or without erlotinib, response rate was lower (11.6%) in patients receiving chemotherapy plus erlotinib compared with patients receiving chemotherapy plus placebo (29.8%; $P = .0495$). Additionally, although PFS was higher with the combination, compared with chemotherapy plus placebo, in FISH-positive patients (6.3 *v* 5.8 months, respectively; $P = .043$), separation of the Kaplan-Meier curve first began 6 months after completion of chemotherapy. Moreover, in FISH-negative patients, PFS numerically favored patients randomly assigned to placebo compared with erlotinib (6 *v* 4.6 months, respectively; $P = .0895$).[30] These results suggest a negative interaction between erlotinib and concurrent chemotherapy not seen in the current S0342 analysis of cetuximab plus chemotherapy.

Lastly, this report does not address other potential predictive EGFR pathway biomarkers (ie, *EGFR* mutations, EGFR protein expression, *KRAS* mutations, and *EGFR* polymorphisms). Although these studies are underway on available S0342 specimens, the tissue resource remaining after *EGFR* FISH analysis is more limited. Results will be reported in conjunction with the overall clinical results of S0342. It is notable, however, that the incidence of *EGFR* FISH positivity in NSCLC populations (35% to 50% in most studies and 59% in the current

analysis) is substantially higher than that of *EGFR* activating mutations in white populations.[8,9,11,12] *EGFR* mutations are typically associated with rapid objective response to EGFR TKIs. However, in the BR.21 study comparing erlotinib with placebo, where the response rate to the EGFR TKI was less than 10%, *EGFR* mutation alone cannot explain the overall survival benefit, much of which is derived from patients who achieve stable disease.[3,12] Furthermore, in TRIBUTE, the presence of *EGFR* mutation correlated with a better patient outcome independent of the therapy administered, indicating a prognostic association rather than a predictive one.[29] In this regard, preclinical studies suggest that response to cetuximab is independent of *EGFR* mutation status.[31] If substantiated in clinical samples, this observation may increase the likelihood that *EGFR* FISH is preferable for selection of NSCLC patients for cetuximab-containing combinations.

In summary, the current study demonstrated improved response, PFS, and overall survival in FISH-positive patients with advanced NSCLC receiving cetuximab-chemotherapy. The median survival time of 15 months in FISH-positive patients is by far the longest survival time achieved in a SWOG trial in this clinical setting and is longer than the median survival time reported with chemotherapy and bevacizumab, which in the United States is considered the current standard of care for patients with advanced NSCLC.[32,33] These findings support the hypothesis that *EGFR* FISH may be broadly applicable for selection of patients for EGFR-targeted therapies. Prospective validation of these results is warranted.

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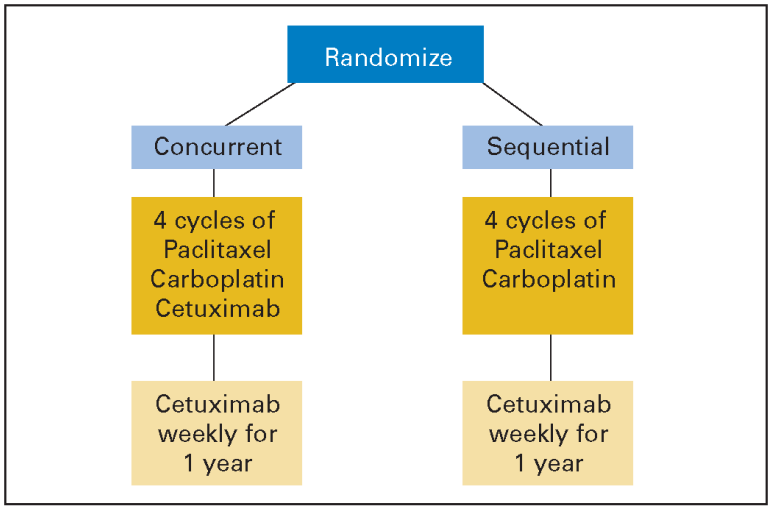

Fig 1.
Phase II study design.

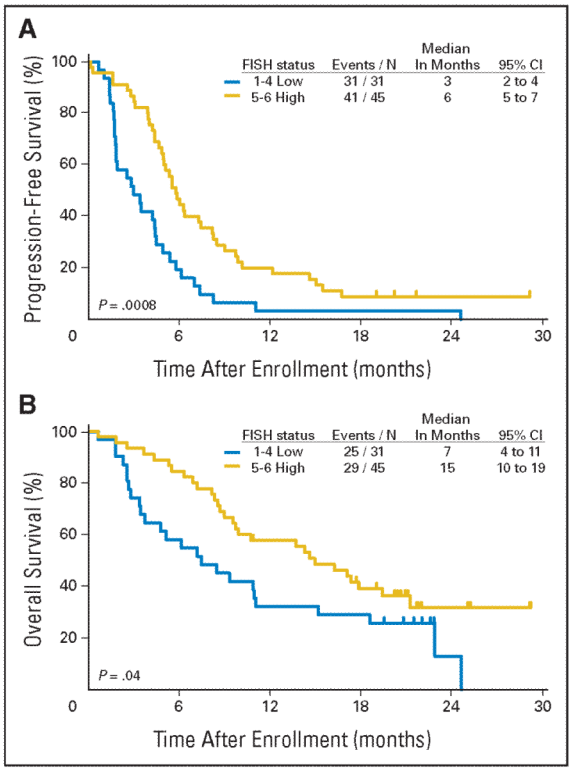

Fig 2.

(A) Progression-free survival and (B) overall survival according to fluorescent in situ hybridization (FISH) status for the entire study population.

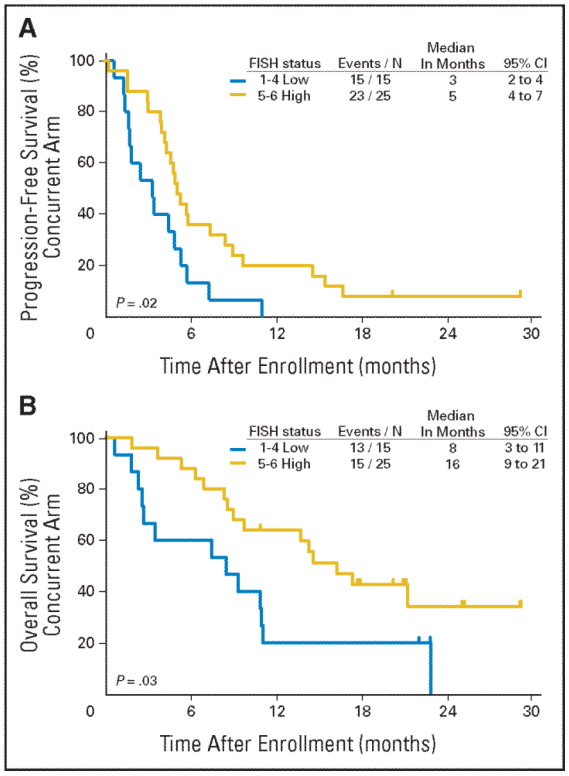

Fig 3.

(A) Progression-free survival and (B) overall survival according to fluorescent in situ hybridization (FISH) status in concurrent treatment arm.

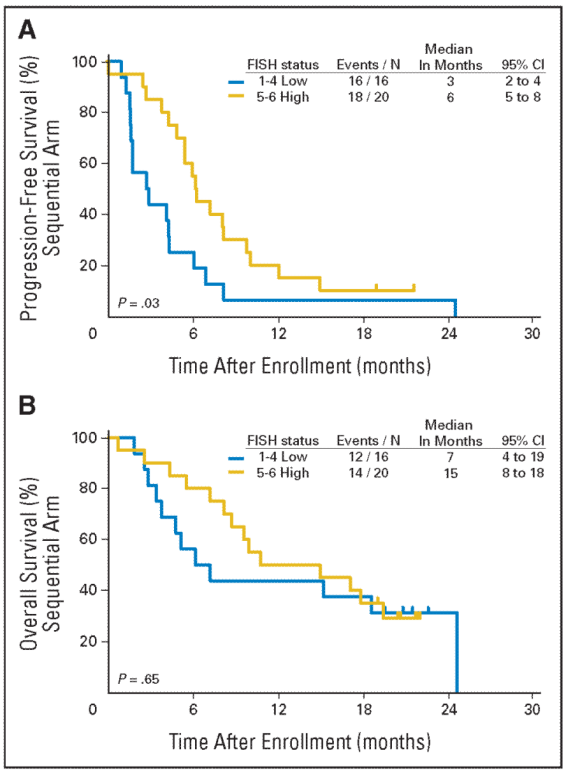

Fig 4.

(A) Progression-free survival and (B) overall survival according to fluorescent in situ hybridization (FISH) status in sequential treatment arm.

Table 1

Patient Characteristics for Patients With Assessable Tissue for *EGFR* FISH Analysis Compared With the Overall Study Population

	FISH-Positive Patients		FISH-Negative Patients		Patiensts With No FISH Results		Total Patients	
Characteristic	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Sex								
Female	25	56	11	35	64	42	100	44
Male	20	44	20	65	89	58	129	56
Race								
White	37	82	24	77	131	86	192	84
Black	5	11	3	10	12	8	20	9
Asian	3	7	3	10	8	5	14	6
Unknown	0	0	1	3	2	1	3	1
Median age, years	63		68		64		64	
Smoking status								
Current	12	27	13	42	65	42	90	39
Former	24	53	14	45	73	48	111	48
Never	9	20	4	13	15	10	28	12
Histology								
Adenocarcinoma	30	67	16	52	84	55	130	57
Squamous cell carcinoma	8	18	9	29	25	16	42	18
Other	7	16	6	19	44	29	57	25
Stage								
IIIB	6	13	4	13	11	7	21	9
IV	39	87	27	87	142	93	208	91

Abbreviations: EGFR, epidermal growth factor receptor; FISH, fluorescent in situ hybridization.

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Table 2

Clinical Outcomes According to FISH Status

Treatment Arm and *EGFR* FISH Status	No. of Patients	OR (%)*	DCR (%)	Median PFS (months)	6-Month PFS Rate (%)	12-Month PFS Rate (%)	HR† for PFS	*P*	Median OS Time (months)	12-Month OS Rate (%)	HR† for OS	*P*
All patients							0.45	.001			0.58	.046
FISH negative	31	26	55	3	19	3			7	32		
FISH positive	45	45	81‡	6	47	20			15	58		
Concurrent arm							0.45	.02			0.43	.03
FISH negative	15	27	53	3	13	0			8	20		
FISH positive	25	42	79	5	36	20			16	64		
Sequential arm							0.46	.03			0.83	.65
FISH negative	16	25	56	3	25	6			7	44		
FISH positive	20	50	83	6	60	20			15	50		
Total population	76	37	70	5	36	13	NA		11	47	NA	

Abbreviations: FISH, fluorescent in situ hybridization; EGFR, epidermal growth factor receptor; OR, overall response (complete plus partial response); DCR, disease control rate; PFS, progression-free survival; HR, hazard ratio; OS, overall survival; NA, not applicable.

*Response evaluation based on overall best response for 73 assessable patients with *EGFR* FISH analysis.

†HRs and *P* values are from univariate Cox proportional hazards models for FISH-positive *v* FISH-negative patients with FISH-negative patients as the reference group.

‡*P* = .02.