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EGFR and HER2 Genomic Gain in Recurrent Non-small Cell Lung Cancer After Surgery:

Impact on Outcome to Treatment with Gefitinib and Association with EGFR and KRAS Mutations in a Japanese Cohort

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Abstract

Background—Sensitivity to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) and frequency of activation mutations in EGFR is lower in Caucasian than Asian non small-cell lung cancer (NSCLC) patients. Increased EGFR gene copy numbers evaluated by fluorescence in situ hybridization (FISH) has been reported as predictor of clinical benefit from EGFR-TKIs in Caucasian NSCLC patients. This study was carried out to verify whether EGFR FISH had similar performance in Japanese patients.

Methods—A cohort of 44 Japanese patients with recurrent NSCLC after surgery was treated with gefitinib 250 mg daily. The cohort included 48% females and 52% never-smokers; 73% had prior chemotherapy and 57% had stage III-IV at the time of surgery. Adenocarcinoma was the most common histology (86%). FISH was performed using the EGFR/Chromosome Enumeration Probe 7 and PathVysion DNA probes (Abbott Molecular). Specimens were classified as FISH positive when showing gene amplification or high polysomy (≥4 copies of the gene in ≥40% of tumor cells). Tumor response to gefitinib was assessed by RECIST for 33 patients with measurable diseases.

Results—Twenty-nine tumors (66%) were EGFR FISH+ and 23 (53%) were HER2 FISH+. Overall response rate was 52%, representing 65% of EGFR FISH+ patients and 29% of EGFR FISH+ patients ($p = 0.0777$). Survival was not impacted by the EGFR FISH ($p = 0.9395$) or the HER2 FISH ($p = 0.0671$) status. EGFR FISH= was significantly associated with HER2 FISH+ ($p = 0.015$) and presence of EGFR mutation ($p = 0.0060$). EGFR mutation significantly correlated with response ($p < 0.0001$) and survival after gefitinib ($p = 0.0204$). EGFR and HER2 FISH status were not associated with KRAS mutation.

Conclusion—Frequency of EGFR FISH+ status was higher and its predictive power for TKI sensitivity was lower in this Japanese cohort than in Western NSCLC cohorts. These findings support differences in the mechanisms of EGFR pathway activation in NSCLC between Asian and Caucasian populations. Confirmation of these results in larger cohorts is warranted.

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Keywords

FISH; EGFR; HER2; KRAS; Biomarkers; NSCLC; Tyrosine inhibitors

Tumor dependence on specific molecular pathways may identify the best target for therapy exploration. Activation of the epidermal growth factor receptor (EGFR)-related signaling pathways drives numerous cancer-promoting processes, such as cell proliferation, apoptosis inhibition, angiogenesis, cell adhesion, and motility and invasion, and also controls development of drug resistance.[1] Therefore, anti-EGFR approaches (antibodies directed against the extracellular domain and small inhibitors of the tyrosine kinase activity) have been one of the most successful examples of molecular target therapy in human solid neoplasias, mainly in non small-cell lung cancer (NSCLC), head and neck, pancreatic and colorectal carcinomas.[2]

Targeted therapies are expected to be effective when the targeted molecule is a major player in the tumor cellular processes, which usually does not occur in all patients with any specific solid tumor. Strategies for patient selection for targeted therapy are almost universally considered to be necessary but are not fully implemented, even for anti-EGFR therapies. In NSCLC, causally associated with EGFR activation are mutations in the adenosine triphosphate-binding site of the tyrosine kinase domain that sustain abnormal response to the ligand,[3,4] activate multiple signaling transduction pathways[5,6] and selectively activate AKT and signal transducers and activators of transcription signaling.[6,7] Increased gene copy numbers is also a well known mechanism for activation of EGFR-related pathways in gliomas,[8] breast,[9] colon,[10] head and neck cancers,[11]and NSCLC.[12]

In NSCLC, at least three molecular markers have been consistently associated with sensitivity or resistance to EGFR-TKIs (tyrosine kinase inhibitors): mutations and amplification/ overrepresentation of the EGFR gene[3–5,12–14] and mutation in the KRAS genes.[15–18] The impact on survival has been extensively investigated for activating EGFR mutations, [19] and less for the EGFR gene copy numbers[12,14,20,21] or for the KRAS mutations[16,22] and results among studies have not been totally concordant. Distinct technologies have been used to identify mutations and genomic gain and part of the discrepancies among results from different studies may due to technical factors. However, other factors such as smoking status, gender, and ethnicity have been demonstrated to impact sensitivity to EGFR-TKIs. Patients of Eastern Asian origin have significantly better clinical outcome to EGFR-TKIs than western populations[23,24] but reasons for these differences are not completely understood. The most important factor so far accounting for this finding is that the Asian NSCLC patients including Japan, have high incidence of activating EGFR mutations.[4,25]

This study aimed to verify the role of EGFR genomic gain as a marker for sensitivity to gefinitib in a Japanese cohort using fluorescence in situ hybridization (FISH), a technology proved to be successful for prediction of outcome to EGFR TKIs in some Caucasian NSCLC populations. In addition, the study aimed to compare EGFR genomic gain with two other gefitinib-related markers, activating mutations in EGFR and resistant mutations in KRAS, which were previously investigated in this cohort.[13]

PATIENTS AND METHODS

Description of Patient Population and Definition of Effectiveness of Gefitinib Treatment

From a population of NSCLC patients who underwent surgery between 1999 and 2003 in the Aichi Cancer Center Hospital in Nagoya, Japan, 75 had recurrent disease and were

treated with 250 mg/daily of gefitinib for recurrent disease. From those, response to treatment could not be evaluated in 6 cases, tumor material was not available in 24 cases, and FISH analyses failed in 4 cases. Thus, the current study reports on 44 patients, all of whom provided consent for the study.

Tumor materials obtained at initial tumor resection for these 44 NSCLC cases had been previously investigated for EGFR and KRAS mutations.[13,16] Tumor response to gefitinib treatment was evaluated for 33 patients eliminating 11 patients who did not have measurable diseases. Tumor response was judged according to the RECIST, without requirement of confirmation of tumor response no less than 4 weeks apart. The length of gefitinib therapy was used as a surrogate for disease free survival and overall survival was calculated form the start of gefitinib administration to death from any cause or the most recent date on which the patient was known to be alive.

EGFR and HER2 Fluorescence In Situ Hybridization Assays

Formalin-fixed, paraffin-embedded tumor blocks were sectioned at 4 μm and submitted to dual-color FISH assays using the Locus Specific Indicator EGFR Spectrum- Orange/CEP 7 SpectrumGreen and the PathVysion DNA Probe Kit (HER2 SpectrumOrange/CEP 17 SpectrumGreen Vysis/Abbott Molecular) following protocol previously described. [12] Briefly, slides were deparaffinized in CitriSolv (Fisher Scientific) and washed in 100% ethanol for 5 minutes. The slides were then sequentially incubated in 2× SSC (saline sodium citrate) at 75°C for 13 to 18 minutes, digested in 0.25 mg/ml Proteinase K/2× SSC at 45°C for 14 to 18 minutes, washed in 2× SSC for 5 minutes and dehydrated in ethanol series. Probes were applied according to the manufacturer instructions to the selected hybridization areas, which were covered and sealed. DNA denaturation was performed in dry oven for 15 minutes at 80°C and hybridization was allowed to occur for 20 hours at 37°C in a humidified chamber. Posthybridization washes were performed consecutively in 2× SSC/ 0.3% NP-40 at 72°C and 2× SSC for 2 minutes each. Following dehydration in ethanol, chromatin was counterstained with 4’ = 6-diamidino-2- phenylindole (DAPI) (0.3 μg/ml in antifade Vectashield mounting medium, Vector Laboratories). Analysis was performed on epifluorescence microscopes using single interference filters sets for green, red (Texas red) and blue (DAPI) as well as dual (red/green) and triple (blue, red, green) band pass filters. For documentation, images were acquired using charged-coupled device camera with Z-stacking and merged using dedicated software (CytoVision, Applied Imaging).

At least 50 tumor nuclei were analyzed in tumor areas selected using the correspondent HE stained slide as a guide. Scoring system followed previous publications.[12] According to the frequency of tumor cells with specific number of copies of the gene and the CEP targets, the tumors were initially classified into six FISH categories (disomy, low and high trisomy, low and polysomy, and gene amplification) and finally grouped into two strata: (a) FISH negative including disomy to low polysomy tumors, which basically have ≥4 copies of the gene in <40% of cells; and (b) FISH positive including tumors with high polysomy (≥4 copies in ≥40% of cells) and gene amplification (defined by a ratio gene/chromosome per cell ≥2, presence of small or nonenumerable clusters of the gene signal or ≥15 copies of the gene signal in ≥10% of the analyzed cells).

Statistical Analysis

For comparisons of proportions, the Pearson’s χ^2 test or the Fisher’s exact test was used. Nonparametric Wilcoxon rank sum test or Kruskal-Wallis test was used to compare the difference in continuous variables. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences between groups were

analyzed by the log-rank test. The two-sided significance level was set at $p < 0.05$. All analyses were performed using SAS version 9.1 (SAS Institute Inc, Cary, NC) software.

RESULTS

Clinical and demographical characteristics are summarized in Table 1. The patients were evenly split between males and females, never or ever smokers and with early or advanced stage disease. Adenocarcinoma histology and poorly or moderately differentiated histologic grade were prevalent. Most patients had not received prior chemotherapy. Median disease free interval after surgery was 375 days, median survival after gefitinib treatment was 562 days, and 66% of patients were alive at the time of last follow up.

EGFR FISH and mutation status in relation to demographics are summarized in Table 2. While EGFR mutation was associated with female gender, never-smoking status, and adenocarcinoma histology, none of these was related with EGFR-FISH status.

Distribution of patients through the FISH categories is illustrated in Figure 1*A* for the EGFR gene and Figure 1*B* for the HER2 gene. The majority of tumors (29 cases [66%]) were EGFR FISH positive, predominantly due to a large representation of tumors with high polysomy (23 cases, 52%, Figure 2*A*) rather than gene amplification (6 cases, 14%, Figure 2*B*). Also, a high number of tumors (23 cases, 53%) were positive for HER2 FISH, of which 21 cases (48%) were represented by high polysomy and only 2 cases (5%) by gene amplification (illustrated in Figure 2*C*). EGFR and HER2 patterns were significantly associated ($p = 0.015$): 19 cases (43%) of tumors were positive and 11cases (25%) were negative for both genes, while 14 cases (32%) had discordant patterns; EGFR FISH positives were more likely to be HER2 FISH positives (19/29 = 66%) than EGFR FISH negatives (4/15 = 27%).

Overall, the specimens with amplification of the EGFR or HER2 genes exhibited clusters of loosely associated signals (Figures 2*B*, *C*) indicating that the amplification occurred as homogenously staining regions. However, one specimen displayed EGFR gene amplification as numerous, diffuse signals mimicking the extrachromosomal double minutes (Figure 2*D*). Heterogeneity for both EGFR and HER FISH patterns was common, with tumor foci showing nuclei with high copy numbers (including gene amplification) interspaced with nuclei with low copy numbers.

The association between FISH patterns and response to the gefitinib treatment for 33 patients with measurable diseases is shown in Table 3. Response to gefitinib was marginally higher in EGFR FISH positive (65%) than negative (29%) patients ($p = 0.0777$). Patients with EGFR gene amplification had a trend towards better benefit (response in 4 of 4 = 100%) than patients with high polysomy (response in 9 of 16 = 56%). HER2 FISH positive pattern trended no impact, including 47% of responders ($p = 0.4426$). Response rate was 62% of patients with EGFR and HER2 FISH positive tumors, in 45% of patients with EGFR or HER2 FISH positive tumors, and in 44% of patients EGFR and HER2 FISH negative tumors. Time to treatment failure (TTF) was not significantly associated with EGFR or HER2 FISH positivity (Table 4). Overall survival was not associated with patterns of EGFR FISH ($p = 0.93$) or HER2 FISH ($p = 0.69$), as shown in Figure 3*A*, *B*. EGFR FISH+ patients with high polysomy (score 5) and true gene amplification (score 6) did not differ regarding survival ($p = 0.6607$; Figure 3*C*).

Among these 44 NSCLC patients, 27 (61%) had activating mutations in the tyrosine kinase domain of the EGFR gene and, among 41 who were tested for KRAS mutations, 5 (12%) had point mutations in codons 12 or 13. Table 3 also shows tumor response according to presence or absence of EGFR and KRAS mutations, both individually and in combination

with EGFR FISH. EGFR mutation was significantly associated with tumor response ($p < 0.0001$) and prolonged TTF ($p < 0.0001$) or survival ($p = 0.02$; Figure 4*A* and Table 4). EGFR FISH positivity was significantly associated with presence of EGFR mutation ($p = 0.0060$). Patients with EGFR mutation were more likely to be EGFR FISH positive (22/27 = 81%) than patients with wild type EGFR (7/17 = 41%). EGFR mutations were present in all 6 tumors with EGFR gene amplification and in 16 out of 23 tumors with EGFR high polysomy (70%). Response rate was 81% of 16 cases positive for both EGFR FISH and mutation and all 4 EGFR FISH negative/EGFR mutation positive cases responded to gefitinib (Table 3).

Conversely, none of the 4 patients with KRAS mutation (none of whom were EGFR FISH positive) or of the 13 patients with EGFR wild type (4 of whom were EGFR FISH positive) benefited from gefitinib treatment. Presence of KRAS mutation was significantly associated with TTF ($p = 0.0248$) but not with lack of response ($p = 0.0995$) or overall survival ($p = 0.4156$, Figure 4*B*).

DISCUSSION

The EGFR FISH positive status had a borderline association to response of gefitinib treatment, but no impact on survival in this cohort of Japanese NSCLC patients. These results do not support a predictive role of the established EGFR FISH assay to gefitinib sensitivity in Japanese NSCLC patients. This observation contrasts with previous findings in Caucasian NSCLC populations obtained by our group[12,20,21] and others,[14] that had identified EGFR genomic gain by FISH as a significant predictor of outcome to EGFR-TKIs. In the current study, EGFR mutation was highly predictive of both response and survival to gefitinib. Lack of predictive value of EGFR FISH or EGFR gene copy numbers as assessed by quantitative polymerase chain reaction have also been reported by Korean[17] and Japanese[26] groups. Therefore, there seems to be ethnic differences as to whether EGFR genomic gain is predictive for response or survival after geftinib treatment.

The clinical and demographical characteristics of this Japanese cohort were distinctive, including high proportion of female, never smokers, early stage disease, no prior chemotherapy, and adenocarcinomas. Unselected cohorts of Asian origin usually have higher frequency of females (40%[27]) and never smokers (40%[27]) than Caucasians (34% for females, 9% for never smokers according to Kobrinsky et al.[28]). In addition, this cohort had one of the highest reported frequencies of EGFR FISH+ tumors (68%) and EGFR mutations (61%). Taken only studies that evaluated gene copy numbers by FISH with identical or similar scoring criteria, the frequency of EGFR FISH+ tumors ranged from 44 to 48% in Asian patients[17,26,29] and from 32 to 45% in Caucasian NSCLCs.[14,21] EGFR activating mutations are well known to be more prevalent in Asian (40–50% of adenocarcinomas[27,30]) than Caucasian NSCLCs (10% of adenocarcinomas[25]). Altogether, these findings substantiate the interesting hypothesis that there are ethnicity-associated molecular peculiarities in NSCLC.

The two EGFR gene markers, activating mutation and genomic gain, were significantly correlated in this cohort. Association between EGFR gene amplification and activating mutations has been reported in NSCLC cell lines[31] and clinical specimens of Caucasian[12] and Asian origins.[17,32] Furthermore, the selective amplification of the mutant allele was verified in the cell lines H3255, H827, PC-9, KT-2, KT-4 and Ma-1,[31] as well as in Asian patients.[32] These findings support the hypothesis that there is a selection of cells carrying the amplification of the mutant allele in lung tumorigenesis. Interestingly, high EGFR copy numbers due to chromosomal aneusomy or structural rearrangements (high polysomy) were also associated with mutations in this cohort and in Caucasian NSCLC.[33]

Status of the HER2 gene in NSCLC has been poorly explored and discrepant results have been reported in association with outcome to EGFR-TKIs.[34] In this cohort, HER2 genomic gain showed up as a negative impact factor for survival after gefinitib treatment, in contrast to our previous results in an Italian cohort.[34] Conversely, none of the five KRAS mutant tumors showed treatment efficacy in this study, in agreement with previously findings that KRAS mutations are primary resistance factors to EGFR-TKIs.[18,35]

In summary, the study showed that the EGFR FISH scoring criteria proposed for stratification of NSCLC for therapy with EGFR-TKIs was not effective in Japanese patients as in Caucasian patients. Confirmation of these results in larger cohorts is warranted and investigation of factors that may underlie distinct molecular mechanisms of activation of the EGFR pathway in these populations should be investigated.

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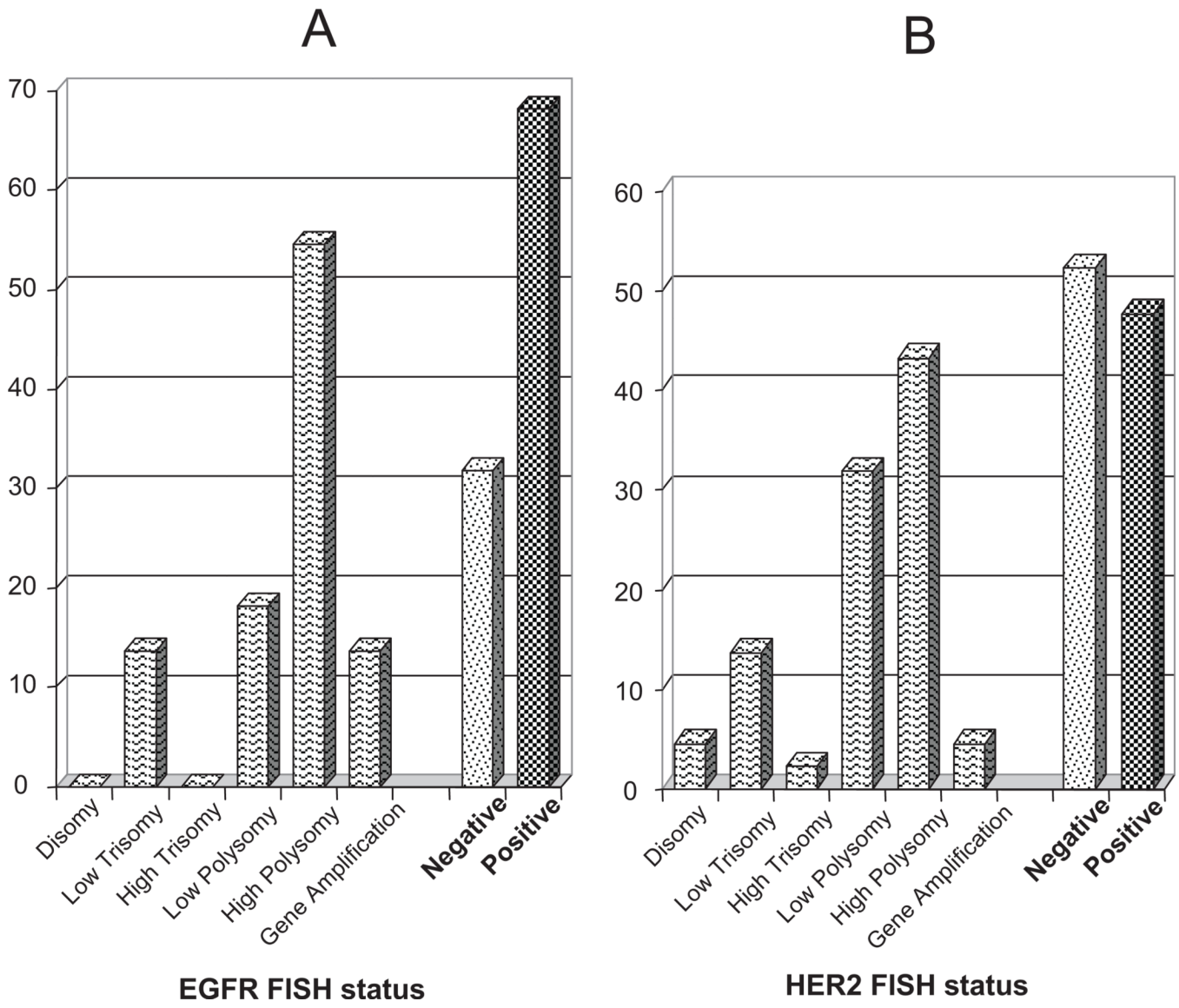

FIGURE 1.

Frequencies of tumors with distinct categories for the epidermal growth factor receptor-fluorescence in situ hybridization (EGFR-FISH) (*A*) and the HER2 FISH (*B*) assays. Negative category includes disomy to low polysomy. Positive category includes high polysomy and gene amplification.

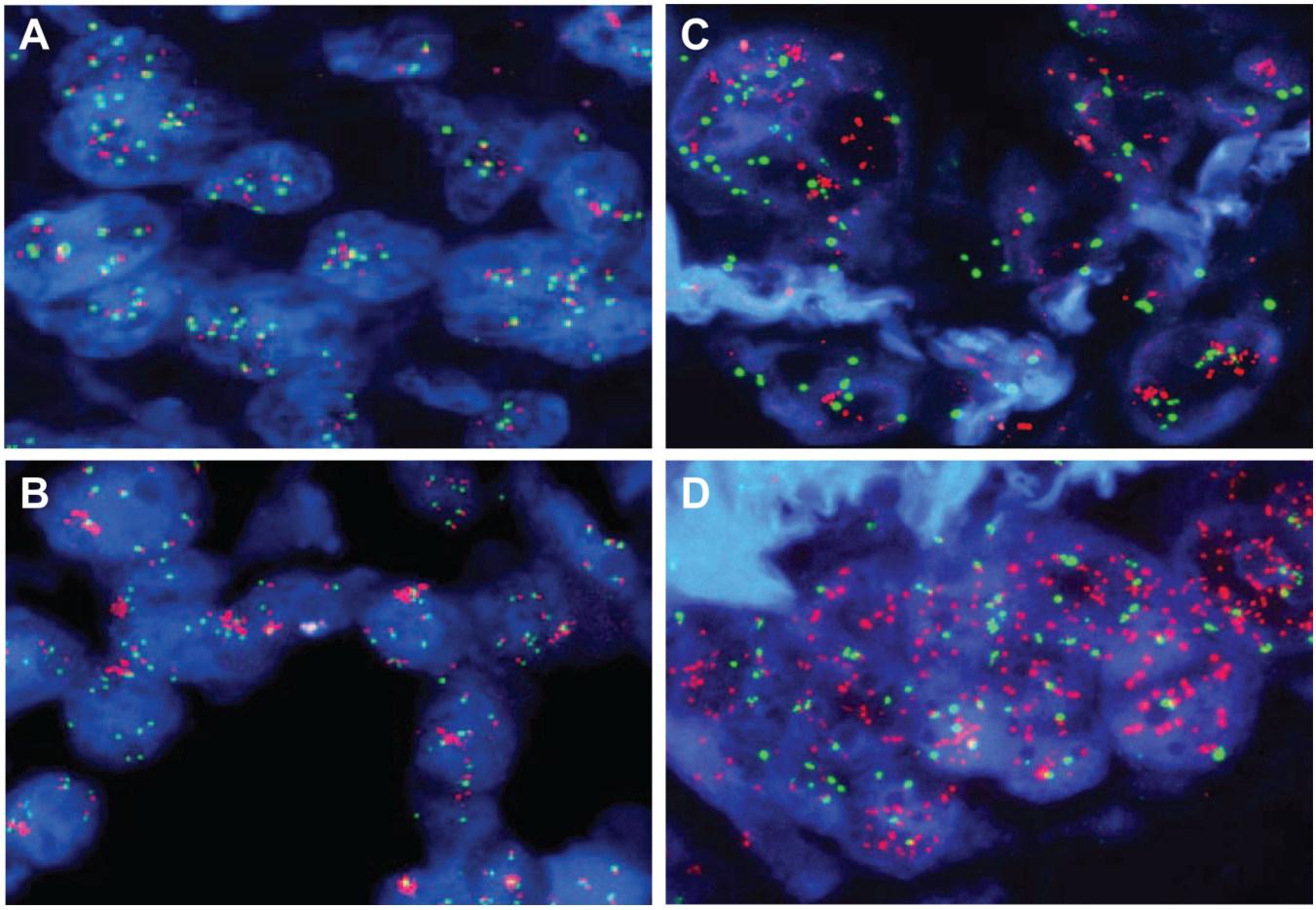

FIGURE 2.

Hybridization of the non small-cell lung cancer (NSCLC) sections with the epidermal growth factor receptor EGFR/CEP7 (*A, B, D*) and the PathVysion probe set (*C*) showing EGFR high polysomy (*A*), EGFR clustered gene amplification (*B*), HER2 gene amplification (*C*) and EGFR amplification as double minutes (*D*).

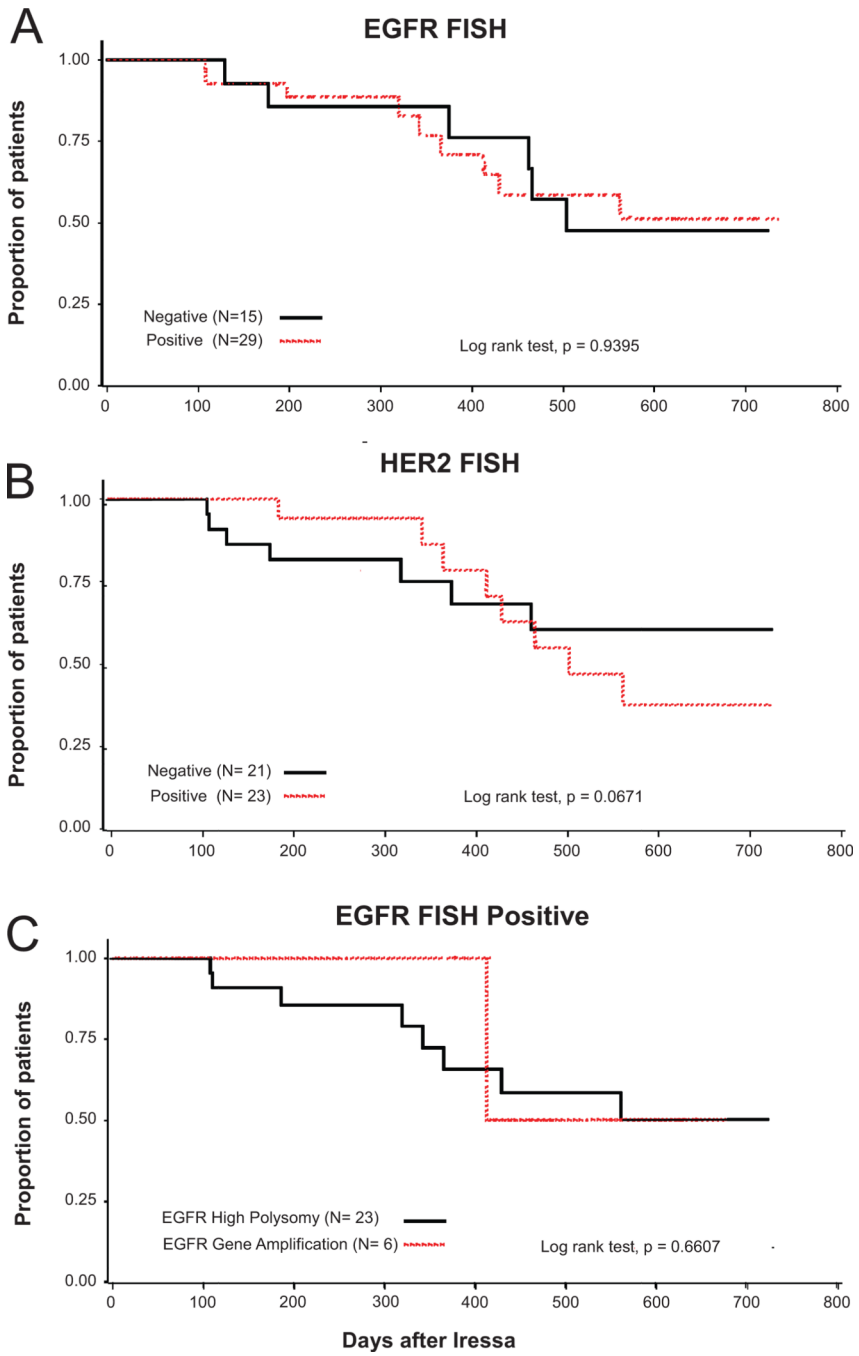

FIGURE 3.

Effect on survival from the day of initiating gefitinib treatment in recurrent non small-cell lung cancer (NSCLC) after surgery by epidermal growth factor receptor fluorescence in situ hybridization (EGFR FISH) status (*A*), HER2 FISH status (*B*), and EGFR high polysomy and gene amplification (*C*).

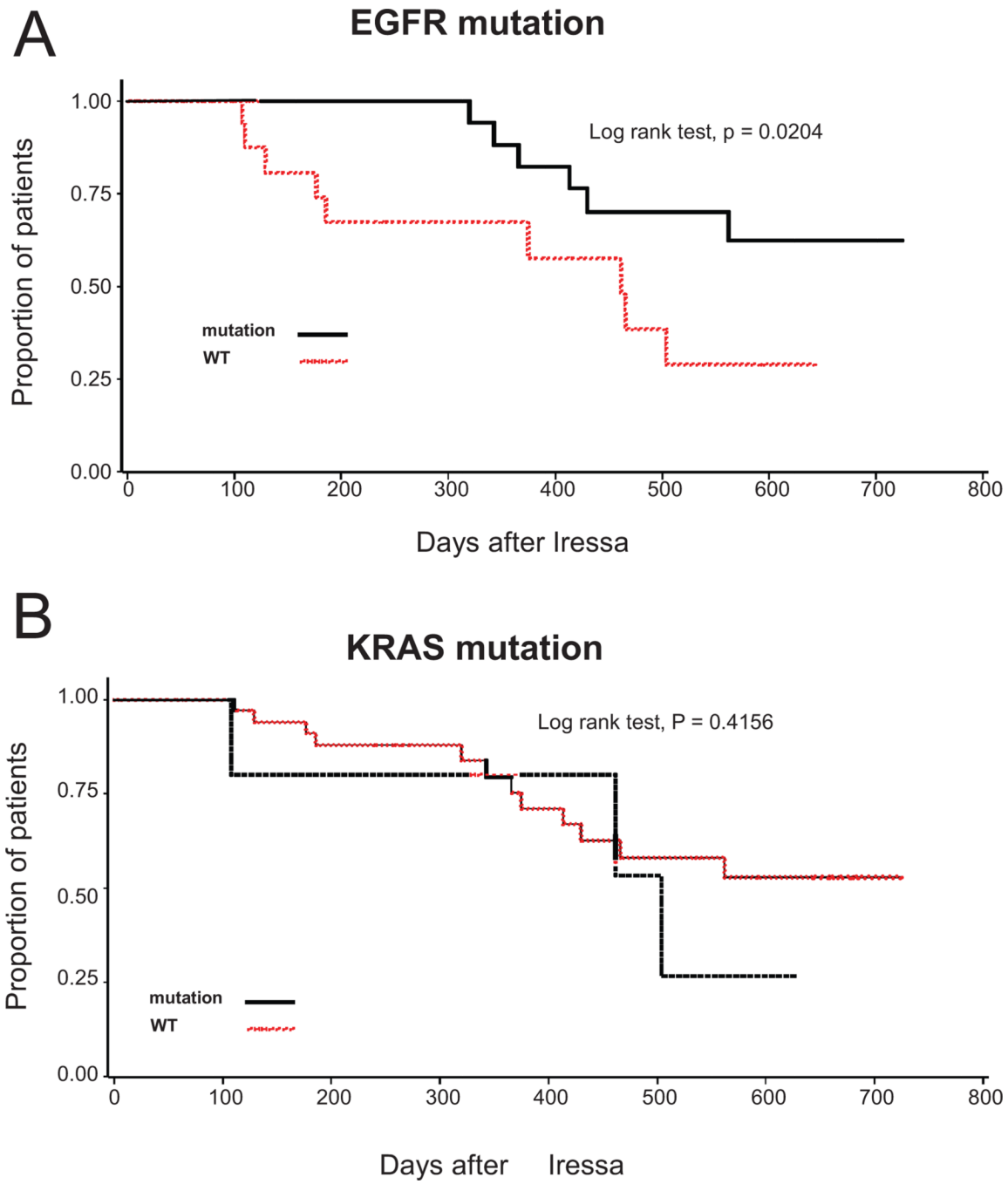

FIGURE 4.

Effect on survival from the day of initiating gefitinib treatment in recurrent non small-cell lung cancer (NSCLC) after surgery by epidermal growth factor receptor (EGFR) activating mutation (*A*) and KRAS mutation (*B*) status.

TABLE 1

Population Characteristics

Variable	Categories	Statistics
Age (years)	Median	60.9 × 10.3
	Range	38–79
Gender	Male	23 (52.3%)
	Female	21 (47.7%)
Smoking	Never	23 (52.3%)
	Ever	21 (47.7%)
Histology	Adenocarcinoma	38 (86.4%)
	Nonadenocarcinoma (SqC, LC)[a]	6 (13.6%)
Differentiation	Poor	10 (26.3%)
	Moderate	22 (57.9%)
	Well	6 (15.8%)
	Not determined	6
Stage	Early (I–II)	19 (43.2%)
	Advanced (III–IV)	25 (56.8%)
Prior chemotherapy	Yes	12 (27.3%)
	No	32 (72.7%)
Survival after surgery (days)	Median	2081
	Range	250–2655
Tumor response (RECIST)	Yes	17 (52%)
	No	16 (48%)
Disease free interval (days)	Median	375
	Range	99–1818
Survival after gefitinib (days)	Median	562
	Range	69–724
Death	Dead	15 (34.1%)
	Alive	29 (65.9%)

[a]SqC, Squamous cell carcinoma; LC, Large cell carcinoma.

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TABLE 2

EGFR FISH and Mutation Status According to Demographics

		EGFR FISH			EGFR Mutation		
Variable	**Categories**	**Positive**	**Negative**	***p***	**Positive**	**Negative**	***p***
Age (years)	Median	61.0	62.0		61.0	61.0	
Gender	Male	15 (65%)	8	$p = 0.9193$	11 (48%)	12	$p = 0.0536$
	Female	14 (67%)	7		16 (76%)	5	
Smoking	Never	15 (67%)	8	$p = 0.9193$	18 (78%)	5	$p = 0.016$
	Ever	14 (65%)	7		9 (43%)	12	
Histology	Adenocarcinoma	25 (66%)	13	$p = 0.9664$	26 (68%)	12	$p = 0.0151$
	Nonadenocarcinoma (SqC, LC)[a]	4 (67%)	2		1 (17%)	5	

FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor.

[a]SqC, Squamous cell carcinoma; LC, Large cell carcinoma.

TABLE 3

Tumor Response in Relation to EGFR FISH, HER2 FISH, EGFR Mutation and KRAS Mutation Status

		Patients		Tumor response			
Molecular marker	**Categories**	***n***	**%**	**PR (%)**	**SD**	**PD**	***p***
EGFR	Positive (+)	20	61	13 (65%)	1	6	$p = 0.0777$
	Negative (−)	13	39	4 (29%)	0	9	
HER2	Positive (+)	17	52	8 (47%)	0	9	$p = 0.4426$
	Negative (−)	16	48	9 (56%)	1	6	
EGFR and HER2	+/+	13	39	8 (62%)	0	5	$p = 0.2451$
	+/−	7	21	5 (71%)	1	1	
	−/+	4	12	0 (0%)	0	4	p^a
	−/−	9	27	4 (44%)	0	5	
EGFR mutation	Positive (+)	20	61	17 (85%)	1	2	$p = 0.0001$
	Negative (−)	13	39	0 (0%)	0	13	
EGFR FISH and EGFR mutation	+/+	16	48	13 (81%)	1	2	$p = 0.0029$
	+/−	4	12	0 (0%)	0	4	
	−/+	4	12	4 (100%)	0	0	p^a
	−/−	9	27	0 (0%)	0	9	
KRAS mutation	Positive (+)	4	13	0 (0%)	0	4	$p = 0.0995$
	Negative (−)	26	87	14 (54%)	1	11	
EGFR FISH and KRAS mutation	+/+	0	0	0 (0%)	0	0	p^a
	+/−	17	57	10 (59%)	1	6	
	−/+	4	13	0 (0%)	0	4	p^a
	−/−	9	30	4 (44%)	0	5	

FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor; PR, partial response; PD, progressive disease.

[a] *p* value could not be calculated because of blank cells.

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TABLE 4

Time to Treatment Failure According to EGFRFISH, HER2 FISH, EGFR Mutation and KRAS Mutation Status

Molecular marker	Categories	Patients		TTF after Gefitinib (Days) Median	*p*
		n	%		
EGFR	Positive (+)	29	66	169	0.722
	Negative (−)	15	34	97	
HER2	Positive (+)	23	53	121	0.1815
	Negative (−)	21	47	144	
EGFR and HER2	+/+	19	43	169	0.0179
	+/−	10	23	118	
	−/+	4	9	56	
	−/−	11	25	144	
EGFR mutation	Positive (+)	27	61	311	<0.0001
	Negative (−)	17	39	83	
EGFR FISH and EGFR mutation	+/+	22	50	182	<0.0001
	+/−	7	16	67	
	−/+	5	11	916	
	−/−	10	23	83	
KRAS mutation	Positive (+)	5	12	87	0.0248
	Negative (−)	36	88	146	
EGFR FISH and KRAS mutation	+/+	1	2	113	0.0767
	+/−	25	61	169	
	−/+	4	10	57	
	−/−	11	25	144	

FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor; TTF, time to treatment failure.