Molecular Alterations in Tumors and Response to Combination Chemotherapy with Gefitinib for Advanced Colorectal Cancer

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Abstract

Purpose: Recently, activating mutations of the epidermal growth factor receptor (*EGFR*) gene were discovered in non–small cell lung cancers sensitive to gefitinib (ZD1839, an EGFR tyrosine kinase inhibitor) but not in gefitinib-resistant cancers. Abnormalities of EGFR and related pathways may have an effect on responsiveness of advanced colorectal cancer to combination chemotherapy with gefitinib.

Experimental Design: We examined patients with previously untreated metastatic colorectal cancer, who were enrolled into two phase I/II trials of combination chemotherapy (irinotecan, leucovorin, and 5-fluorouracil) and daily oral gefitinib. We obtained paraffin tissue blocks of primary tumors from 31 patients, sequenced the *EGFR*, *KRAS*, and *BRAF* genes, and did immunohistochemistry for EGFR, phosphorylated AKT1, p53, p21, and p27.

Results: Twelve (39%) of the 31 patients experienced a partial objective response to the therapy. A novel *EGFR* mutation in exon 18 (c.2170G>A, p.Gly724Ser) was identified in only one patient who did not experience an objective tumor response. EGFR immunohistochemistry was not predictive of responsiveness. In contrast, loss of p21 was associated with a higher response rate to therapy ($P = 0.05$). Moreover, the response rate among patients whose tumors maintained p21 expression and possessed a mutation in p53 was only 9% (1 of 11, $P = 0.005$). Overexpression of phosphorylated AKT1 also seemed to predict a trend towards resistance to the therapy.

Conclusions: p21 expression in colorectal cancer, especially in combination with p53 mutation, is a predictor of resistance to the combination chemotherapy with gefitinib. Activating *EGFR* mutations are rare in colorectal cancer and do not seem to confer sensitivity to gefitinib and chemotherapy.

The epidermal growth factor receptor (encoded by the *EGFR* gene, OMIM #131550) tyrosine kinase mediates cellular responses to growth signals through RAS, signal transduction and activator of transcription (STAT), and protein kinase C pathways. Inhibition of EGFR by either oral tyrosine kinase inhibitors or monoclonal antibodies has shown tumor regression in patients with lung and colorectal adenocarcinomas (1). Activating mutations of *EGFR* have been discovered in non–small cell lung cancers (NSCLC) that were sensitive to EGFR tyrosine kinase inhibitor gefitinib (ZD1839, "Iressa") but not in gefitinib-resistant cancers (2, 3). These discoveries have opened possibilities for targeted therapies against other tumors with activating *EGFR* mutations, as well as against tumors with alterations in downstream events that might predict responsiveness to EGFR-directed therapy. NSCLC cells expressing mutant EGFR undergo extensive apoptosis after small interfering RNA against mutant *EGFR* or treatment with pharmacologic inhibitors of AKT and STAT signaling but are relatively resistant to conventional chemotherapeutic agents (4), emphasizing the importance of the EGF/AKT/STAT pathways in NSCLC. In contrast, mutations in *KRAS* have been shown to predict resistance of lung adenocarcinoma to gefitinib and erlotinib (5).

Depending on methods of detection, ~50% to 80% of human colorectal cancers have been shown to express EGFR, suggesting that EGFR represents an attractive target in colorectal cancer patients (6, 7). Phase II trials of monoclonal antibodies directed against EGFR's extracellular domain have shown objective tumor responses in patients with colorectal cancer (8–10). Among patients with advanced colorectal cancer, a combination of gefitinib with chemotherapy showed a 78% response rate among chemotherapy-naive patients and 36% among those who had progressed through first-line therapy (11). A number of combinations of radiation and/or

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chemotherapy with agents directed against EGFR have been under investigation either *in vitro* or *in vivo* (12–20).

Unlike NSCLC, it remains unclear whether activating mutations in *EGFR* have a role in colorectal carcinogenesis. *EGFR* mutations were identified in 4 of 33 (12%) patients with colorectal cancer in Japan (21), although in other studies, an *EGFR* mutation was identified in only 1 of 293 (0.34%) colorectal cancer cases (22) and in none of 20 colorectal cancer cases (2). Moreover, no study to date has examined the influence of *EGFR* mutations on the responsiveness to gefitinib-based therapy in advanced colorectal cancer. We therefore investigated the influence of *EGFR* mutations and protein expression as well as other events related to downstream pathways [including alterations of *KRAS*, *BRAF*, p21 (CDKN1A/CIP1), p27 (CDKN1B/KIP1), p53 (TP53), and phosphorylated AKT1] on the treatment response among patients who were enrolled in phase I/II trials of gefitinib and chemotherapy for chemotherapy-naive metastatic colorectal cancer.

Materials and Methods

Patient selection and treatment. Patients for this analysis were enrolled into one of two phase I/II trials that examined the maximum tolerated dose of daily oral gefitinib in combination with i.v. irinotecan, leucovorin, and 5-fluorouracil (5-FU) in previously untreated metastatic colorectal cancer. The study has been approved by the Dana-Farber Harvard Cancer Center Institutional Review Board. All patients provided written informed consent, permitting us to do genomic/genetic analysis on their samples, before participation in the clinical trials. In both trials, patients had histologically confirmed metastatic colorectal cancer. Prior therapy for metastatic disease was not permitted, and all patients had measurable disease based on the Response Evaluation Criteria in Solid Tumors (23). Eligibility criteria also included the following: age of ≥18; Eastern Cooperative Oncology Group performance status of 0 to 2; life expectancy of ≥12 weeks; adequate hematopoietic function (absolute neutrophil count of ≥1,500/mm^3, platelets ≥ 100,000/mm^3); and adequate renal (serum creatinine ≤ 1.5 mg/dL) and hepatic function (serum bilirubin ≤ 1.5 mg/dL).

Patients in the first trial (gefitinib plus bolus IFL) received daily oral gefitinib in combination with irinotecan 100 (or 125) mg/m^2, leucovorin 20 mg/m^2, and 5-FU 400 (or 500) mg/m^2 delivered i.v. weekly for two consecutive weeks followed by 1-week rest. In the second trial (gefitinib plus FOLFIRI), daily oral gefitinib was given with irinotecan 180 mg/m^2 i.v. bolus, leucovorin 200 mg/m^2 i.v. bolus, 5-FU 400 mg/m^2 i.v. bolus followed by 5-FU 2400 mg/m^2 via continuous infusion over 46 hours; chemotherapy was repeated every 2 weeks. Therapy was continued until disease progression, unacceptable toxicity, or withdrawal of patient consent. In both trials, doses of oral gefitinib were started at 250 mg/d and the dose was escalated to 500 mg/d in cohorts of three patients. In both studies, the maximum tolerated dose of gefitinib was 250 mg/d.

Assessment of objective tumor response. In each trial, computed axial tomographic scans were done at baseline and every 6 weeks thereafter. Response evaluation was assessed according to the Response Evaluation Criteria in Solid Tumors (23).

Tumor tissue samples and genomic DNA extraction. Of 35 patients enrolled in the two trials, primary tumor specimens were available from 31 patients. Paraffin-embedded tissue blocks were obtained at the time of resection of the primary tumor. Tumor tissue (and normal tissue if applicable) was reviewed and dissected from tissue sections obtained from the paraffin tissue blocks, and genomic DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Valencia, CA).

PCR and sequencing of EGFR, KRAS, and BRAF. For whole genome amplification, genomic DNA was PCR amplified using random 15-mer primers (24, 25). Each PCR mix contained 20 pmol of the random primers, 1.69 nmol each of deoxynucleotide triphosphate, 2.5 mmol/L of $MgCl_2$, 1× PCR buffer (Applied Biosystems, Foster City, CA), 0.5 unit of AmpliTaq Gold (Applied Biosystems), and 2 μL of template DNA solution in a total volume of 27 μL. PCR condition consisted of initial denaturing at 94°C (1 minute); 50 cycles of 94°C (30 seconds), 37°C (2 minutes), 41.5°C (30 seconds), 46°C (30 seconds), 50.5°C (30 seconds), 55°C (2 minutes), and 68°C (30 seconds); and final extension at 72°C (2 minutes) (25). PCR of *EGFR* was targeted for exons 18, 19, and 21 in the critical kinase domain. PCR Primers for *EGFR*, *KRAS* (exon 1), and *BRAF* (exon 15) were EGFR-E18F, 5′-TTGTGGAGCCTCTTACACCC-3′; EGFR-E18R, 5′-ATGAGAGGCCCTGCGGCCCA-3′; EGFR-E19F, 5′-TGCCAGTTAACGTCTTCCTTC-3′; EGFR-E19R, 5′-GGGCCTGAGGTTCAGAGCCA-3′; EGFR-E21F, 5′-ACCGTCGCTTGGTGCACCGC-3′; EGFR-E21R, 5′-GTCAGGAAAATGCTGGCTGA-3′; KRAS-F14, 5′-TGTAAAACGACGGCCAGTTGTGTGACATGTTCTAATATAGTCAC-3′; KRAS-R7, 5′-AGAATGGTCCTGCACCAGTAA-3′; BRAF-F7, 5′-TCCTTTACTTACACCTC-3′; and BRAF-R7, 5′-AAATAGCCTCAATTCTTACC-3′. Each PCR mix contained the forward and reverse primers (each 10 pmol), 1.69 nmol each of deoxynucleotide triphosphate, 3 mmol/L of $MgCl_2$, 1× PCR buffer, 0.75 unit of AmpliTaq Gold, and 2 μL of template whole genome amplification product in a total volume of 27 μL. PCR condition consisted of initial denaturing at 94°C (1 minute); 50 cycles of 95°C (20 seconds), annealing (20 seconds; 48°C for *BRAF*, 50°C for *KRAS*, 52°C for *EGFR* exon 19, 55°C for *EGFR* exons 18 and 21), and 72°C (40 seconds); and final extension at 72°C (2 minutes). The PCR products were purified using QIAquik PCR Purification Kit (Qiagen), cycle-sequenced with BigDye Terminator kit (Applied Biosystems), and analyzed by ABI 3730 (Applied Biosystems). All forward sequencing results were confirmed by reverse sequencing. *KRAS* sequencing was further validated by Pyrosequencing (25). A mutation and polymorphism in *EGFR* were further confirmed by a duplicated run from the original DNA samples. By our sequencing methods, we could detect *EGFR* mutations in NSCLC cell lines 3255 (with the p.Leu858Arg mutation in exon 21) and H1650 (exon 19 deletion mutation; both provided by Matthew Meyerson, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA).

Immunohistochemical analyses. For EGFR immunohistochemistry, paraffin sections of colorectal cancers were deparaffinized, incubated with 3% H_2O_2 (20 minutes) to block endogenous peroxidase, and incubated with pepsin at 37°C (10 minutes). Protein block (Vector Laboratories, Burlingame, CA; 20 minutes) was followed by application of primary anti-EGFR antibody (Zymed Laboratories, South San Francisco, CA; dilution 1:100; overnight at 4°C). Then, secondary anti-mouse antibody (Vector Laboratories) was applied (20 minutes), avidin biotin complex was added, and sections were visualized by diaminobenzidine (5 minutes) and methyl-green counterstain. EGFR expression was recorded as negative (0), weakly positive (1+), positive (2+), or strongly positive (3+).

For phosphorylated AKT1 (p-AKT1) immunohistochemistry, antigen retrieval was done by incubating deparaffinized tissue sections in citrate buffer by a microwave at 92.8°C for 15 and 30 minutes. Tissue sections were then incubated with 3% H_2O_2 (30 minutes) to block endogenous peroxidase and incubated with protein block (Vector Laboratories; 30 minutes). Primary anti-p-AKT1 antibody (Cell Signaling, Beverly, MA; dilution 1:50) was applied for overnight at 4°C. Then, secondary anti-rabbit antibody (Vector Laboratories) was applied (30 minutes), avidin biotin complex was added, and sections were visualized by 3,3′-diaminobenzidine (5 minutes) and methyl-green counterstain. p-AKT1 expression was interpreted as negative or positive, using normal epithelial cells and lymphocytes as reference.

For p21 (CDKN1A/CIP1) and p27 (CDKN1B/KIP1) immunohistochemistry, antigen retrieval was done by incubating deparaffinized tissue sections in citrate buffer at high power in a microwave for 30 minutes (in a pressure cooker) and 15 minutes, respectively. Tissue sections were then incubated with 3% H_2O_2 (10 minutes) to block endogenous peroxidase and incubated with protein block (Vector Laboratories; 10 minutes). Primary anti-p21 antibody (PharMingen,

San Diego, CA; dilution 1:50) or anti-p27 antibody (Transduction Laboratories, San Diego, CA; dilution 1:200) was applied for 30 minutes at room temperature. Then, biotinylated secondary Multi-Link antibody (Biogenex, San Ramon, CA) was applied (20 minutes), horseradish peroxidase avidin complex (Biogenex) was added, and sections were visualized by 3,3′-diaminobenzidine (30 seconds) and methyl-green counterstain. p21 expression was recorded as negative (<10% of cells staining) or positive (≥10% of cells staining). p27 expression was recorded as negative (≤20% of cells staining) or positive (>20% of cells staining).

We assessed for mutations in p53 (*TP53*) by immunohistochemistry. Methods for p53 immunohistochemistry were previously described (26). Only strong and unequivocal staining with ≥10% of cells staining was interpreted as positive. Weak or no staining for p53 was regarded as negative.

All immunohistochemical and molecular results were interpreted as blinded from patients' identity and clinical outcomes data.

Statistical analysis. Statistical analysis was done using the SAS program (version 9.1, SAS Institute, Cary, NC). Given the limited sample size of patient population, Fisher's exact test was used for the analysis on categorical data. Statistical significance was set at $P = 0.05$. All P values are two sided.

Results

Clinical and pathologic characteristics. Primary tumor specimens were obtained from 31 evaluable patients enrolled in two trials assessing the efficacy and safety of a combination of gefitinib with irinotecan, leucovorin, and 5-FU in previously untreated metastatic colorectal cancer. All 31 patients (22 men and 9 women) were Caucasians with age ranging from 31 to 83 years (median, 57 years). Clinical characteristics of these patients are listed in Table 1. Forty-three percent and 57% of patients had Eastern Cooperative Oncology Group performance status of 0 and 1, respectively, and Eastern Cooperative Oncology Group performance status was not a predictor of chemoresponsiveness. All 31 patients had measurable disease by the Response Evaluation Criteria in Solid Tumors; 12 patients (39%) experienced a partial response as their best response; 16 patients (52%) experienced stable disease; and one patient (3%) experienced only progressive disease. Responsiveness to therapy was not associated with pathologic features such as tumor differentiation, or presence of mucinous or signet ring cell component (data not shown).

Epidermal growth factor receptor mutation and protein expression. Of 31 available tumor specimens from patients treated with gefitinib and chemotherapy, we successfully sequenced exons 18, 19, and 21 in 30 specimens (Table 1). Of the 30 colorectal cancer tumors analyzed for gene mutations, we found one novel mutation of *EGFR*, c.2170G>A (p.Gly724Ser; Fig. 1A-B), in a tumor with wild-type *KRAS* and *BRAF*. This mutation resides in exon 18, the critical kinase domain of *EGFR*. We confirmed the presence of this mutation by duplicating all steps of the PCR-sequencing assays and excluded the possibility of a germ line polymorphism by sequencing normal DNA from the same patient. This c.2170G>A (p.Gly724Ser) mutation in the kinase domain confers a glycine-to-serine substitution at codon 724. Notably, a previously described mutation at codon 719 (c.2155G>A, p.Gly719Ser) also confers a glycine-to-serine substitution and has been associated with gefitinib sensitivity in NSCLC (3). However, in the current study, the patient whose colorectal cancer possessed the c.2170G>A (p.Gly724Ser) mutation did not experience any disease regression following treatment with gefitinib and chemotherapy; the patient achieved stable disease for only 6 months before developing disease progression. In a second patient, we found a germline polymorphism, c.2203G>A (p.Ala735Thr), in exon 18, and confirmed its presence in both the tumor and normal tissue (Fig. 1C). Interestingly, in this tumor, the normal allele seemed deleted to create a loss of heterozygosity pattern. However, this second patient also did not experience any disease regression following treatment with gefitinib and chemotherapy and achieved stable disease for only 6 months before developing disease progression. No other patient was found to harbor a somatic *EGFR* exon 18, 19, or 21 mutation.

We also examined tumor expression of EGFR as measured by immunohistochemistry (Table 2). Of the 27 tumors for which EGFR expression could be evaluated, 12 (44%) exhibited 2+ or 3+ (strong) EGFR expression and 11 (41%) exhibited 1+ (weak) expression. However, levels of EGFR expression were not associated with tumor responsiveness to gefitinib and chemotherapy ($P = 0.45$; Table 2).

KRAS and BRAF status and response to therapy. We considered the possibility that responsiveness to gefitinib and chemotherapy may reflect other somatic events in the tumors. Among the 30 tumors that were sequenced for mutations in *KRAS*, we observed mutations in 10 patients [33%; four were c.38G>A (codon 13 GAC; p.Gly13Asp), three c.34G>A (codon 12 AGT; p.Gly12Ser), two c.35G>A (codon 12 GAT; p.Gly12Asp), and one c.35G>T (codon 12 GTT; p.Gly12Val)]. Moreover, using sequence analysis, we found two cases with mutations in *BRAF* [6.7%; both were c.1799T>A (p.Val600Glu; previously called as "V599E") mutations]. Of note, we found no significant association between *KRAS* status (Table 2) or *BRAF* status and response to gefitinib and chemotherapy.

Expression of p21 (CDKN1A/CIP1), p27 (CDKN1B/KIP1), p53, and phosphorylated AKT1 and response to therapy. We examined alterations in events that are potentially downstream from EGFR, including p21, p27, p53, and p-AKT1. Loss of p21 expression was found in 43% of cases and was associated with a higher response rate to therapy; 67% of cases with p21 loss experienced an objective tumor response, whereas 25% of cases with p21 positivity responded to therapy ($P = 0.05$; Table 2). Furthermore, we observed a striking trend towards resistance to therapy among patients whose tumors maintained p21 expression and had a mutation in p53. Only 1 of 11 (9%) patients with p21 expression and mutated p53 experienced a partial response, whereas 11 of 16 (69%) patients with either p21 loss or wild-type p53 responded to therapy (P for comparison = 0.005; Table 2). With regard to p-AKT1, 14% of tumors with p-AKT1 overexpression responded to therapy, whereas 50% of tumors without p-AKT1 responded, although this difference did not reach a level of statistical significance ($P = 0.19$). In contrast, expression of p27 was not related to response to therapy.

Discussion

Recent research efforts have found that activating mutations in the EGFR kinase domain represent an important predictor for responsiveness to gefitinib in NSCLC (2, 3). These specific activating mutations have been limited to exons 18, 19, and 21, which are in the tyrosine kinase domain of EGFR (2, 3).

Table 1. Clinical and molecular features of advanced colorectal cancer treated with combination chemotherapy including gefitinib

Case no.	Age/Sex	Gefitinib dose (mg)	Chemotherapy	Best response to therapy	*EGFR*	*KRAS*	*BRAF*	EGFR IHC*	p-AKT1 IHC	p53 IHC	p21 IHC	p27 IHC
1	67/F	250	IFL[†]	PR	WT	c.38G>A	WT	3	N	N	N	N
2	52/F	500	IFL[‡]	S	WT	WT	WT	2	N	P	P	N
3	39/M	500	IFL[‡]	S	WT	c.38G>A	WT	2	N	N	P	N
4	66/F	250	IFL[‡]	PR	WT	c.38G>A	WT	2	N	N	P	P
5	51/M	250	IFL[‡]	PR	WT	WT	WT	1	N	N	P	P
6	59/M	500	IFL[‡]	Not evaluable	WT	WT	c.1799T>A	3	N	P	P	P
7	57/M	250	IFL[‡]	S	c.2170G>A	WT	WT	1	N	P	P	P
8	68/M	250	IFL[‡]	PR	WT	WT	WT	2	N	P	N	P
9	56/M	250	IFL[‡]	PR	WT	WT	WT	2	N	P	N	P
10	49/M	500	IFL[‡]	S	WT	WT	WT	1	N	P	P	P
11	58/M	250	IFL[‡]	PR	WT	WT	WT	1	P	N	P	P
12	45/M	250	IFL[‡]	PR	WT	WT	WT	—	N	P	N	P
13	46/M	250	IFL[‡]	PR	WT	c.38G>A	WT	2	N	P	N	N
14	64/M	500	IFL[‡]	S	WT	c.35G>A	WT	0	P	P	N	P
15	62/F	500	FOLFIRI[§]	S	WT	c.34G>A	WT	1	N	P	P	P
16	31/M	250	FOLFIRI[§]	S	WT	WT	WT	1	N	P	N	P
17	41/M	250	FOLFIRI[§]	S	WT	c.34G>A	WT	1	P	P	P	P
18	38/M	250	FOLFIRI[§]	PR	WT	WT	WT	1	N	P	N	P
19	46/M	500	FOLFIRI[§]	S	WT	WT	WT	0	N	P	—	P
20	83/M	250	FOLFIRI[§]	Not evaluable	WT	c.35G>T	WT	2	N	P	P	P
21	63/M	250	FOLFIRI[§]	PR	WT	WT	WT	2	N	P	N	P
22	41/M	500	FOLFIRI[§]	S	WT	WT	WT	0	P	P	P	N
23	67/M	250	IFL[†]	S	c.2203G>A germline polymorphism	WT	WT	1	N	P	P	P
24	57/F	250	FOLFIRI[§]	S	WT	WT	WT	2	P	P	P	P
25	60/M	250	FOLFIRI[§]	PR	WT	WT	WT	1	N	N	N	P
26	66/F	250	FOLFIRI[§]	S	WT	c.34G>A	WT	2	P	P	P	N
27	38/F	250	FOLFIRI[§]	S	WT	c.35G>A	WT	2	N	P	P	N
28	43/F	250	IFL[‡]	S	—	—	—	2	N	N	N	N
29	59/M	250	FOLFIRI[§]	PD	WT	WT	c.1799T>A	—	P	—	P	N
30	51/M	250	FOLFIRI[§]	PR	WT	WT	WT	1	N	P	P	N
31	79/F	250	FOLFIRI[§]	S	WT	WT	WT	0	N	P	N	N

NOTE: —, no result.
Abbreviations: IHC, immunohistochemistry; IFL, bolus 5-FU, leucovorin, and irinotecan; PR, partial response; WT, wild-type; N, negative; S, stable disease; P, positive; FOLFIRI, infusional 5-FU, leucovorin, and irinotecan; PD, progressive disease.
*IHC results are 0, negative; 1, weak positive; 2, positive; and 3, strong positive.
[†]5-FU 500 mg/m^2, leucovorin 20 mg/m^2, irinotecan 125 mg/m^2, days 1 and 8 of a 21-day cycle.
[‡]5-FU 400 mg/m^2, leucovorin 20 mg/m^2, irinotecan 100 mg/m^2, days 1 and 8 of a 21-day cycle.
[§]5-FU bolus 400 mg/m^2, leucovorin 200 mg/m^2, irinotecan 180 mg/m^2, infusional 5-FU 2.4 g/m^2 over 46 hours, repeated every 2 weeks.

Activating *EGFR* mutations selectively activate AKT and STAT signaling pathways (4). NSCLC cells expressing mutant EGFR undergo extensive apoptosis after small interfering RNA against mutant *EGFR* or treatment with pharmacologic inhibitors of AKT and STAT signaling but are relatively resistant to conventional chemotherapeutic agents (4).

Lynch et al. (2) sequenced *EGFR* exons 19 and 21 but not exon 18, in colorectal cancer cell lines (COLO-205, HCT-116, HCT-15, HT-29, and SW-620) and found no mutations. Barber et al. (22) similarly identify only one *EGFR* mutation (p.Gly719Ser) in 293 cases of colorectal cancer. In contrast, Nagahara et al. (21) recently identified somatic *EGFR* mutations in 4 of 33 (12%) cases of colorectal cancer. Our results confirm the rarity of activating *EGFR* mutation in colorectal cancer; we found one novel mutation (c.2170G>A, p.Gly724Ser) in exon 18 of *EGFR* (in the critical kinase domain). This mutation is particularly interesting because it represents an identical amino acid substitution (glycine to serine) as another well-described activating mutation (c.2155G>A, p.Gly719Ser; refs. 3, 22), which is only five amino acids away from the currently described mutation. Although functional analysis is lacking in this study, it remains a possibility that the p.Gly724Ser mutation represents an activating *EGFR* mutation given the importance of the glycine-to-serine substitution at codon 719. Nonetheless, in contrast to NSCLC, this *EGFR* mutation was identified in a patient who did not experience an objective

tumor response to gefitinib and chemotherapy. In addition, EGFR expression, as measured by immunohistochemistry, was not associated with responsiveness to the chemotherapy with gefitinib. Studies of cetuximab (a monoclonal antibody to EGFR) in colorectal cancer has similarly failed to show a relation between EGFR expression and response to therapy (8).

Although several studies suggest that a monoclonal antibody to EGFR can result in tumor regression in patients with colorectal cancer (8–10), as a single agent, oral tyrosine kinase inhibitors such as gefitinib seem less active (27). The potential diminished activity of oral tyrosine kinase inhibitors in colorectal cancer may reflect the rarity of activating *EGFR* mutations in colorectal cancer, although other mechanisms of resistance may exist. Of note, if *EGFR* mutations are uncommon in colorectal cancer, other downstream events such as alterations in p-AKT1 or p21 may influence responsiveness to EGFR inhibition.

We observed a significantly lower response to gefitinib and chemotherapy among patients whose tumors had intact p21 expression. Moreover, the prediction for treatment resistance was most striking in patients whose tumors maintained p21 expression in the setting of mutated p53. Still, we cannot exclude the possibility that the inverse relation between p21 expression and response to gefitinib and chemotherapy was principally related to the effect of 5-FU and irinotecan rather than any interaction with gefitinib. However, in contrast to our findings, a recent review of 1,809 colorectal cancer cases in the published literature suggest that, overall, greater p21 expression predicted a greater responsiveness to fluoropyrimidine-based chemotherapy that did not include an EGFR inhibitor (28). We are unaware of any other study that has examined predictors of responsiveness to gefitinib-based therapy in patients with colorectal cancer. Previous studies have shown that overexpression of p21-activated kinase-1 (PAK1) is associated with greater metastatic potential in colorectal cancer (29) and that gefitinib can suppress PAK1 pathway and the subsequent invasiveness of cancer cells (30). Taken together, these data could suggest that p21 overexpression may overcome the inhibitory effect of gefitinib through activation of PAK1 pathway. Gefitinib has been shown to up-regulate p27 and p21 in head and neck squamous cell cancer cells, and suppression of p21 and p27 by antisense constructs can decrease the growth inhibitory effect of gefitinib (31), indicating the important roles of p21 and p27 in modulating the effect of gefitinib.

Previous studies have examined the influence of p53 and p-AKT1 status on interventions that inhibit EGFR. MDM2 antisense oligonucleotides, which potentiate p53 activity, enhance the effect of EGFR inhibitors by affecting *in vitro* and *in vivo* proliferation, apoptosis, and protein expression in hormone-refractory and hormone-dependent human prostate cancer cells (32). Moreover, combination of gefitinib and antisense MDM2 synergistically inhibits the growth of hormone-independent prostate cancer cells, and this effect is accompanied by the inhibition of p-AKT1 (32), suggesting a

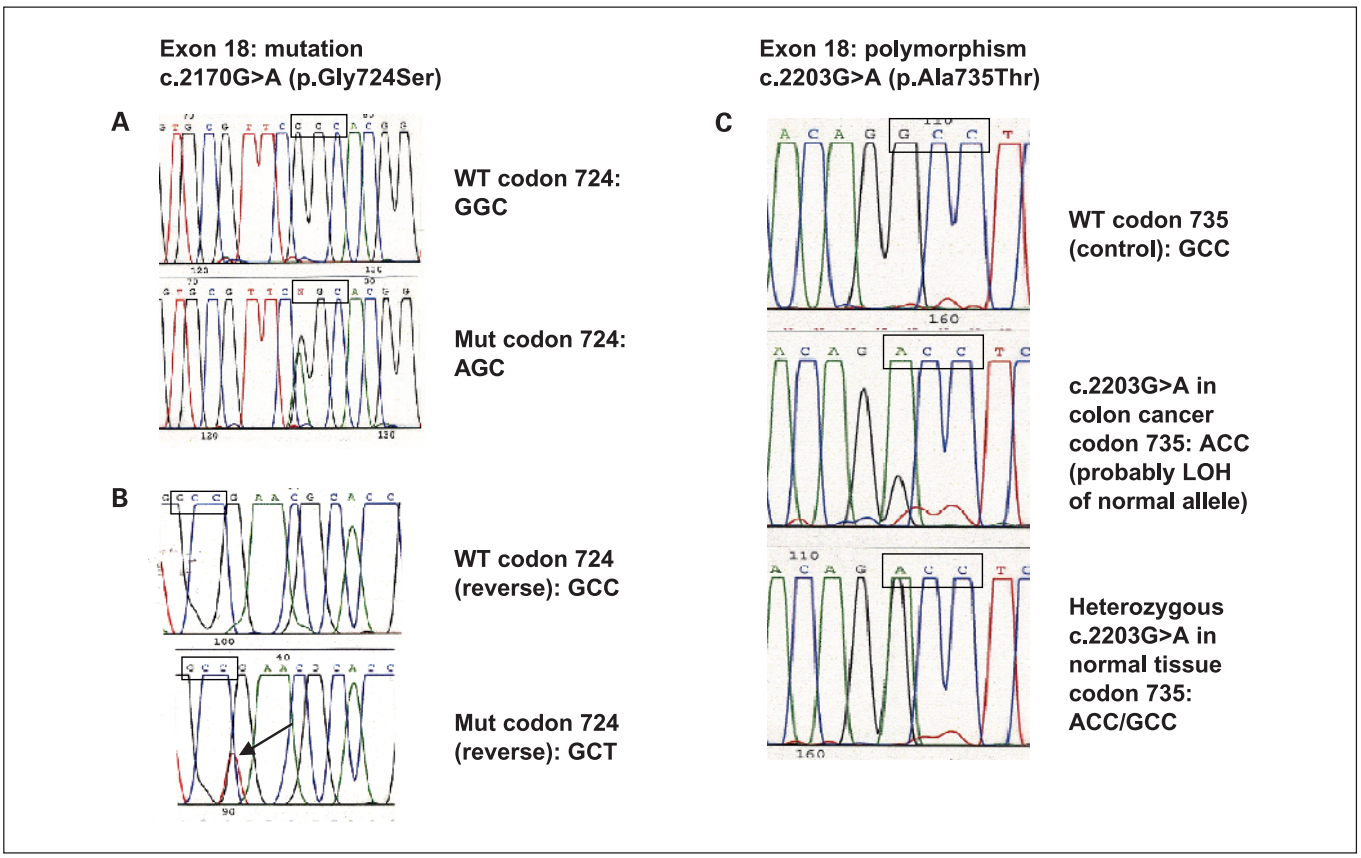

Fig. 1. *EGFR* sequencing. *A,* c.2170G>A (p.Gly724Ser) mutation (Mut) in colon cancer (*bottom*). Wild-type (WT) normal tissue (*top*). *B,* reverse sequence of the c.2170G>A mutation. *C,* c.2203G>A (p.Ala735Thr) polymorphism is shown in normal tissue as heterozygous (*bottom*). In colon cancer, the normal allele appears to be deleted creating a loss of heterozygosity (*LOH*) pattern (*middle*).

Table 2. Molecular markers and response to the combination chemotherapy with gefitinib

Molecular marker	No. of patients	Response rate* (%)	*P*
EGFR expression			0.12
Positive	23	48	
Negative	4	0	
KRAS mutation			0.69
Mutant	9	33	
Wild-type	19	47	
p21 expression†			0.05
Positive	16	25	
Negative	12	67	
p53 mutation‡			0.10
Positive	21	33	
Negative	7	71	
p21† and p53‡			0.005
Both positive	11	9.1	
Either one or both negative	16	69	
p-AKT1 expression			0.19
Positive	7	14	
Negative	22	50	
p27 expression§			0.25
Positive	17	53	
Negative	11	27	

*Response rate is defined as the fraction of cases with partial response among the total number of cases with a particular molecular feature and known response status.
†p21 positivity is defined as ≥10% of tumor cells staining.
‡p53 mutational status was determined by immunohistochemistry.
§p27 positivity is defined as >20% of tumor cells staining.

role for intact p53 in augmenting the effect of gefitinib. Our results are consistent with these previous data; we observed that loss of functional p53 limited the response to gefitinib and chemotherapy, particularly in tumors with intact p21.

With regard to p-AKT1, previous studies have shown that response to gefitinib is associated with p-AKT1 overexpression in NSCLC (33–36). In contrast, our results showed a trend towards gefitinib sensitivity in p-AKT1-negative colorectal cancer. However, different mechanisms likely underlie sensitivity to EGFR inhibition in colorectal cancer than those observed in NSCLC. Gefitinib can down-regulate p-AKT1 or up-regulate p27 expression in some patients with metastatic colorectal cancer when pretreatment and posttreatment tumor specimens are compared (37). Further investigation is necessary to confirm the effect of p53 and p-AKT1 on gefitinib sensitivity in colorectal cancer.

In conclusion, activating *EGFR* mutations are rare in colorectal cancer and do not seem to confer responsiveness to gefitinib and chemotherapy. Nonetheless, p21 expression in colorectal cancer, especially in combination with p53 mutation, seems a predictor of resistance to the combination of chemotherapy with gefitinib. Further studies of EGFR inhibitors in colorectal cancer should examine the role of such downstream events on treatment efficacy.

Note Added in Proof

Microsatellite instability was assessed in 28 patients, and all 28 tumors showed stable microsatellites of BAT25, BAT26, and BAT40 markers. Methods of microsatellite instability analysis are described in Ogino et al. (38).

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References

1. El-Rayes BF, LoRusso PM. Targeting the epidermal growth factor receptor. Br J Cancer 2004;91:418–24.
2. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
3. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
4. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
5. Pao W, Wang TY, Riely GJ, et al. KRAS mutations and primary resistance to lung adenocarcinomas to gefitinib or erlotinib. PLoS Med 2005;2:e17.
6. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183–232.
7. Dannenberg AJ, Lippman SM, Mann JR, Subbaramaiah K, DuBois RN. Cyclooxygenase-2 and epidermal growth factor receptor: pharmacologic targets for chemoprevention. J Clin Oncol 2005;23:254–66.
8. Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004;351:337–45.
9. Chung KY, Shia J, Kemeny NE, et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 2005;23:1803–10.
10. Saltz LB, Meropol NJ, Loehrer PJ, Sr., et al. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 2004;22:1201–8.
11. Fisher GA, Kuo T, Cho CD, et al. A phase II study of gefitinib in combination with FOLFOX-4 (IFOX) in patients with metastatic colorectal cancer [abstract]. J Clin Oncol 2004;22:3514.
12. Bianco C, Tortora G, Bianco R, et al. Enhancement of antitumor activity of ionizing radiation by combined treatment with the selective epidermal growth factor receptor-tyrosine kinase inhibitor ZD1839 (Iressa). Clin Cancer Res 2002;8:3250–8.
13. Tortora G, Caputo R, Damiano V, et al. Combination of a selective cyclooxygenase-2 inhibitor with epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 and protein kinase A antisense causes cooperative antitumor and antiangiogenic effect. Clin Cancer Res 2003;9:1566–72.
14. Mantha AJ, Hanson JE, Goss G, et al. Targeting the mevalonate pathway inhibits the function of the epidermal growth factor receptor. Clin Cancer Res 2005;11:2398–407.
15. Prewett MC, Hooper AT, Bassi R, et al. Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (CPT-11) against human colorectal tumor xenografts. Clin Cancer Res 2002;8:994–1003.
16. Tortora G, Caputo R, Damiano V, et al. Combined targeted inhibition of bcl-2, bcl-XL, epidermal growth factor receptor, and protein kinase A type I causes potent antitumor, apoptotic, and antiangiogenic activity. Clin Cancer Res 2003;9:866–71.
17. Ciardiello F, Caputo R, Damiano V, et al. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. Clin Cancer Res 2003;9:1546–56.
18. Nyati MK, Maheshwari D, Hanasoge S, et al. Radiosensitization by pan ErbB inhibitor CI-1033 *in vitro* and *in vivo*. Clin Cancer Res 2004;10:691–700.
19. Ciardiello F, Bianco R, Caputo R, et al. Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. Clin Cancer Res 2004;10:784–93.
20. Tuccillo C, Romano M, Troiani T, et al. Antitumor activity of ZD6474, a vascular endothelial growth factor-2 and epidermal growth factor receptor small molecule tyrosine kinase inhibitor, in combination with SC-236, a cyclooxygenase-2 inhibitor. Clin Cancer Res 2005;11:1268–76.
21. Nagahara H, Mimori K, Ohta M, et al. Somatic mutations of epidermal growth factor receptor in colorectal carcinoma. Clin Cancer Res 2005;11:1368–71.
22. Barber TD, Vogelstein B, Kinzler KW, Velculescu VE. Somatic mutations of EGFR in colorectal cancers and glioblastomas. N Engl J Med 2004;351:2883.
23. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the

United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.

24. Dietmaier W, Hartmann A, Wallinger S, et al. Multiple mutation analyses in single tumor cells with improved whole genome amplification. Am J Pathol 1999;154:83–95.

25. Ogino S, Kawasaki T, Brahmandam M, et al. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 2005;7:413–21.

26. Reles A, Schmider A, Press MF, et al. Immunostaining of p53 protein in ovarian carcinoma: correlation with histopathological data and clinical outcome. J Cancer Res Clin Oncol 1996;122:489–94.

27. Mackenzie MJ, Hirte HW, Glenwood G, et al. A phase II trial of ZD1839 (Iressa™) 750 mg per day, an oral epidermal growth factor receptor-tyrosine kinase inhibitor, in patients with metastatic colorectal cancer. Invest New Drugs 2005;23:165–70.

28. Geller JI, Szekely-Szucs K, Petak I, Doyle B, Houghton JA. P21Cip1 is a critical mediator of the cytotoxic action of thymidylate synthase inhibitors in colorectal carcinoma cells. Cancer Res 2004;64:6296–303.

29. Carter JH, Douglass LE, Deddens JA, et al. Pak-1 expression increases with progression of colorectal carcinomas to metastasis. Clin Cancer Res 2004;10:3448–56.

30. Yang Z, Bagheri-Yarmand R, Wang RA, et al. The epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa) suppresses c-Src and Pak1 pathways and invasiveness of human cancer cells. Clin Cancer Res 2004;10:658–67.

31. Di Gennaro E, Barbarino M, Bruzzese F, et al. Critical role of both p27KIP1 and p21CIP1/WAF1 in the antiproliferative effect of ZD1839 ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor, in head and neck squamous carcinoma cells. J Cell Physiol 2003;195:139–50.

32. Bianco R, Caputo R, Damiano V, et al. Combined targeting of epidermal growth factor receptor and MDM2 by gefitinib and antisense MDM2 cooperatively inhibit hormone-independent prostate cancer. Clin Cancer Res 2004;10:4858–64.

33. Amann J, Kalyankrishna S, Massion PP, et al. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. Cancer Res 2005;65:226–35.

34. Cappuzzo F, Magrini E, Ceresoli GL, et al. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2004;96:1133–41.

35. Ono M, Hirata A, Kometani T, et al. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. Mol Cancer Ther 2004;3:465–72.

36. Han SW, Hwang PG, Chung DH, et al. Epidermal growth factor receptor (EGFR) downstream molecules as response predictive markers for gefitinib (Iressa, ZD1839) in chemotherapy-resistant non-small cell lung cancer. Int J Cancer 2005;113:109–15.

37. Daneshmand M, Parolin DA, Hirte HW, et al. A pharmacodynamic study of the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 in metastatic colorectal cancer patients. Clin Cancer Res 2003;9:2457–64.

38. Ogino S, Brahmandam M, Cantor M, et al. Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. Mod Pathol 2005; In press.

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