

EGFR Fluorescence *In situ* Hybridization Pattern of Chromosome 7 Disomy Predicts Resistance to Cetuximab in KRAS Wild-type Metastatic Colorectal Cancer Patients

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Abstract

Purpose: Metastatic colorectal cancer patients with low epidermal growth factor receptor (*EGFR*) gene copy number are unlikely to respond to anti-*EGFR* monoclonal antibody (mAb) treatment. The objective of this study was to investigate *EGFR* fluorescence *in situ* hybridization (FISH) patterns of chromosome 7 disomy with efficacy of cetuximab therapy in metastatic colorectal cancer patients.

Experimental Design: We detected the *EGFR* FISH patterns and *KRAS* status in 74 tumors from cetuximab-treated metastatic colorectal cancer patients and analyzed with response rate (RR) and progression-free survival (PFS).

Results: One of the 16 (6.25%) patients with chromosome 7 homogeneous disomy (defined as FISH negative) had objective response to cetuximab. A total of 53 (76.8%) patients with chromosome 7 pattern of variable ratios of disomy versus polysomy (defined as FISH positive) had a significantly higher RR (37.7% versus 6.25%; $P = 0.01$), a trend towards longer PFS (4.5 versus 2.9 months; $P = 0.07$). Among 54 *KRAS* wild-type patients, *EGFR* FISH-positive patients had significantly higher RR (51.3% versus 9%; $P = 0.01$) and longer PFS (5.0 versus 2.3 months; $P = 0.02$) than *EGFR* FISH-negative patients. However, among 20 *KRAS* mutant-type patients, there was no difference in RR (0% versus 0%) and PFS (2.5 versus 3.8 months; $P = 0.51$) between *EGFR* FISH-positive and -negative patients.

Conclusion: Our results show firstly that patients with *EGFR* FISH pattern of chromosome 7 disomy have a very low chance to benefit from cetuximab-based therapy. *EGFR* FISH pattern of chromosome 7 disomy may be as a negative predicative factor for cetuximab response in *KRAS* wild-type metastatic colorectal cancer patients. *Clin Cancer Res*; 17(2); 382–90. ©2011 AACR.

Introduction

It has been shown that the anti-epidermal growth factor receptor (anti-*EGFR*) monoclonal antibody (mAb) cetuximab (Erbix, developed by Merck KGaA) as monotherapy or for combination with chemotherapy can improve responsiveness and prolong survival in patients with metastatic colorectal cancer (1–4), but only 10% to 20% of patients respond to this agent. Several recent clinical studies have

shown that the presence of a *KRAS* mutation is a significant predictor of resistance to anti-*EGFR* mAbs (5–7). On the basis of this finding, the European Union drug regulatory body, the European Medicines Agency, has approved the use of anti-*EGFR* mAbs only for metastatic colorectal cancer patients whose tumors display wild-type (WT) *KRAS*. However, the occurrence of *KRAS* mutations only accounts for approximately 30% to 40% of nonresponsive patients. Therefore, the identification of additional genetic determinants of treatment benefit still needs to be defined.

Recently, studies have suggested that an increased *EGFR* gene copy number (GCN) analyzed by the fluorescence *in situ* hybridization (FISH) technique could be a promising predictor of anti-*EGFR* mAb therapy in metastatic colorectal cancer (8–11). Patients with low GCN are indeed unlikely to respond to anti-*EGFR* mAb treatment and have less progression-free time than patients with increased GCN. However, the *EGFR* FISH pattern of metastatic colorectal cancer is often not homogeneous, and has variable ratios of disomy versus polysomy or amplification. In these situations, the definition of *EGFR* patterns and the reproducibility of data lead to difficulties in direct comparison and clinical application. Moroni et al. reported that chromosome 7 homogeneous disomy is the most frequent

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Translational Relevance

Metastatic colorectal cancer patients with low epidermal growth factor receptor (*EGFR*) gene copy number (GCN) are unlikely to respond to anti-*EGFR* monoclonal antibody treatment. However, the definition of GCN and the reproducibility of data lead to the difficulties in clinical application. In this article, we describe the *EGFR* FISH patterns of chromosome 7 homogeneous disomy, which is the most frequent pattern of nonincreased *EGFR* GCN in colorectal cancer and is easy to detect as a negative predicative factor for cetuximab response in *KRAS* wild-type metastatic colorectal cancer. Together with *KRAS* mutation, chromosome 7 disomy may predict more patients who will not respond to cetuximab.

pattern in metastatic colorectal cancer with decreased *EGFR* GCN (12). They suggested that chromosome 7 disomy is easier to detect than an increase in *EGFR* copy number and therefore, might enable a more reproducible FISH result. However, no clinical data have supported his hypothesis yet. In addition, *EGFR* is a transmembrane tyrosine kinase receptor that, on ligand binding, mainly triggers the RAS-RAF-MAPK and PI3K-PTEN-AKT signaling pathways. The resistance to anti-*EGFR* mAbs may be due to constitutive activation of the downstream genes of the *EGFR* signaling pathway such as *KRAS*, *BRAF*, or *PIK3C2A*, or to the loss of a tumor suppressor gene such as *PTEN*. This implies that pathways rather than single genes should be the focus of studies aimed at analyzing anti-*EGFR* mAb therapy.

The aim of the present study was therefore to examine *EGFR* FISH patterns combined with *KRAS* mutation status in metastatic colorectal cancer patients, and investigate their associations with response to cetuximab therapy. To this end, we first evaluated whether previously generated cutoff points could be validated in our independent series. Second, we assessed whether chromosome 7 disomy could be used as *EGFR* FISH result criteria on this data set. Furthermore, we explored the combination of *EGFR* GCN with *KRAS* status which currently is the best-established marker for outcome prediction after cetuximab is administered for colorectal cancer.

Patients and Methods

Patients

This retrospective study enrolled 74 consecutive metastatic colorectal cancer patients treated with cetuximab-containing regimens between May 2005 and March 2010 from three institutions in China, including Sun Yat-sen University Cancer Center (Guangzhou), Beijing Cancer Hospital (Beijing), and The First People's Hospital of Foshan (Guangdong Province). Patients were selected based on the following criteria: histologically proven metastatic colorectal adenocarcinoma; presence of at least one measurable lesion; cetuximab-containing regimens

were received after failure of irinotecan- and/or oxaliplatin-based regimens; sufficient specimens of formalin-fixed paraffin-embedded tissue were available from primary colorectal and/or metastatic tumors; never previously received *EGFR*-targeted therapy; having signed informed consent form. The study was approved by the Research Ethics Committee of the Sun Yat-sen University Cancer Center (reference YP-2009177).

Cetuximab was administered as a loading dose of 400 mg/m² i.v., followed by a dose of 250 mg/m² once a week. All patients received cetuximab in combination with cytotoxic drugs; 63 (85.1%) patients received cetuximab plus irinotecan or irinotecan-based chemotherapy, 10 (13.5%) received cetuximab plus oxaliplatin-based chemotherapy, and 1 (1.4%) received cetuximab plus capecitabine chemotherapy.

Clinical response was assessed every 6 to 8 weeks by radiologic examination (computed tomography or magnetic resonance imaging). The Response Evaluation Criteria in Solid Tumors guidelines (13) were adopted for evaluation, and objective tumor response was classified as complete response, partial response, stable disease, or progressive disease. Patients with complete response or partial response were defined as responders, whereas patients with stable disease or progressive disease were defined as non-responders. Progression-free survival (PFS) was calculated from the time of first cetuximab infusion to the time of disease progression or death from any cause. Overall survival (OS) was calculated from the time of first cetuximab infusion to patient death or last contact.

DNA extraction and *KRAS* mutation analysis

DNA was extracted from paraffin-embedded colorectal cancer samples using the QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's recommendations after a histologic control for the presence of tumor cells (>70%) in each tumor sample. A real-time PCR genotyping method was done for the detection of *KRAS* codon 12 and codon 13 mutations. The presence of *KRAS* mutations (6 at codon 12 and 1 at codon 13) was determined by allelic discrimination assay on an ABI 7900HT Sequence Detection System (Applied Biosystems). Specific probes for each allele (mutated and wild alleles) were labeled with the fluorescence reporter dyes FAM or VIC at their 5'-end. Briefly, reactions were done in a 25 μ L mixture comprising 50 ng of DNA, 0.2 μ L (20 μ mol/L) of specific primers and probes, and 12.5 μ L \times TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR amplification was done under the following cycle conditions: 95 °C for 10 minutes; 40 cycles, 95 °C for 30 seconds; and 60 °C for 1 minute. Data were analyzed with SDS2.0 software (Applied Biosystems). Each mutation detected by allelic discrimination was validated by direct sequencing analysis.

Determination of *EGFR* gene copy number by FISH

EGFR GCN per cell was investigated by FISH using the LSI *EGFR* Spectrum Orange/CEP 7 Spectrum Green probe

(Vysis, Abbott Laboratories) according to the manufacturer's protocol. Briefly, 2- μ m-thick tissue sections were cut and incubated at 56 °C overnight; after being deparaffinized and dehydrated, the sections were incubated in 2 \times saline sodium citrate buffer (2 \times SSC; pH 7.0) at 75 °C for 20 minutes. Then the sections were digested with proteinase K (0.2 mg/mL in 2 \times SSC, pH 7.0) at 37 °C for 20 minutes, rinsed in 2 \times SSC (pH 7.0) at room temperature for 5 minutes, fixed in 10% neutral buffered formalin, and dehydrated using ethanol in a series of increasing concentrations (70%, 85%, 100%). The probe sets were applied onto the tissue areas on each slide, and the hybridization area was covered with a glass coverslip and sealed with rubber cement. The slides were incubated in a humidified atmosphere at 85 °C for 5 minutes for codenaturation of probe and target DNA, and subsequently at 37 °C for 16 hours for hybridization. Posthybridization washes were done in 1.5 mol/L Urea and 0.1 \times SSC (pH 7.0–7.5) at 45 °C for 30 minutes and in 2 \times SSC for 2 minutes at room temperature. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). FISH signals for each locus-specific FISH probe were assessed under an Olympus BX51 TRF microscope (Olympus, Japan) equipped with a triple-pass filter (DAPI/green/orange; Vysis).

Without knowledge of the patients' clinical molecular characteristics, two independent observers (WF and SQ) scored at least 100 nonoverlapping interphase nuclei for the number of copies of *EGFR* and *CEP7* by use of predefined scoring guidelines. The negative controls consisted of a healthy colorectal mucosa adjacent to malignant disease; the control for amplified *EGFR* was an amplified colonic adenocarcinoma. FISH patterns were defined as described in (14, 15): Briefly, the samples were grouped as follows: normal disomy, ≤ 2 gene copies in $>90\%$ of cells; trisomy, 3 gene copies in $>10\%$ of cells and ratio gene/chromosomes ≤ 2 ; low polysomy, ≥ 4 gene copies in $>10\%$ but $<40\%$ of cells and ratio gene/chromosomes ≤ 2 ; high polysomy, ≥ 4 gene copies in $>40\%$ cells and ratio gene/chromosomes ≤ 2 ; and gene amplification, ratio gene/chromosome >2 or 15 gene copies in $\geq 10\%$ of cells. Trisomy, low polysomy, high polysomy, and/or gene amplification were considered *EGFR*-FISH positive. Normal disomy was considered *EGFR*-FISH negative.

Statistics

Differences in response rate (RR) were tested by the Fisher's exact test; PFS, OS, and the 95% confidence intervals (95% CI) were evaluated by Kaplan-Meier survival analysis. Comparisons of PFS and OS between different groups were done by the log-rank test. *EGFR* sensitivity and specificity were expressed in terms of percentage, and the value for which sensitivity and sensibility were the highest was chosen as the best cutoff point. All statistical analyses were carried out on SPSS 13.0 software and $P < 0.05$ was considered statistically significant.

Table 1. Characteristics of 74 patients of metastatic colorectal cancer patients

| Patient characteristics | Number (%) |
|--------------------------------|------------|
| Total evaluated | 74 (100) |
| Gender | |
| Male | 43 (58.1) |
| Female | 31 (41.9) |
| Age (year) | |
| Median | 53 |
| Range | 23–82 |
| Primary tumor site | |
| Colon | 43 (58.1) |
| Rectum | 31 (41.9) |
| Previous chemotherapy regimens | |
| Irinotecan containing | 59 (79.7) |
| Oxaliplatin containing | 69 (93.2) |
| Cetuximab treatment line | |
| First line | 0 |
| Second line | 15 (20.3) |
| Third line and more | 59 (79.7) |
| Treatment regimens | |
| Cetuximab monotherapy | 0 |
| Cetuximab plus chemotherapy | 74 (100) |
| Treatment duration (weeks) | |
| Mean | 11 |
| Range | 2–38 |
| Kras mutation status | |
| Wild-type | 54 (73) |
| Mutation | 20 (27) |

Results

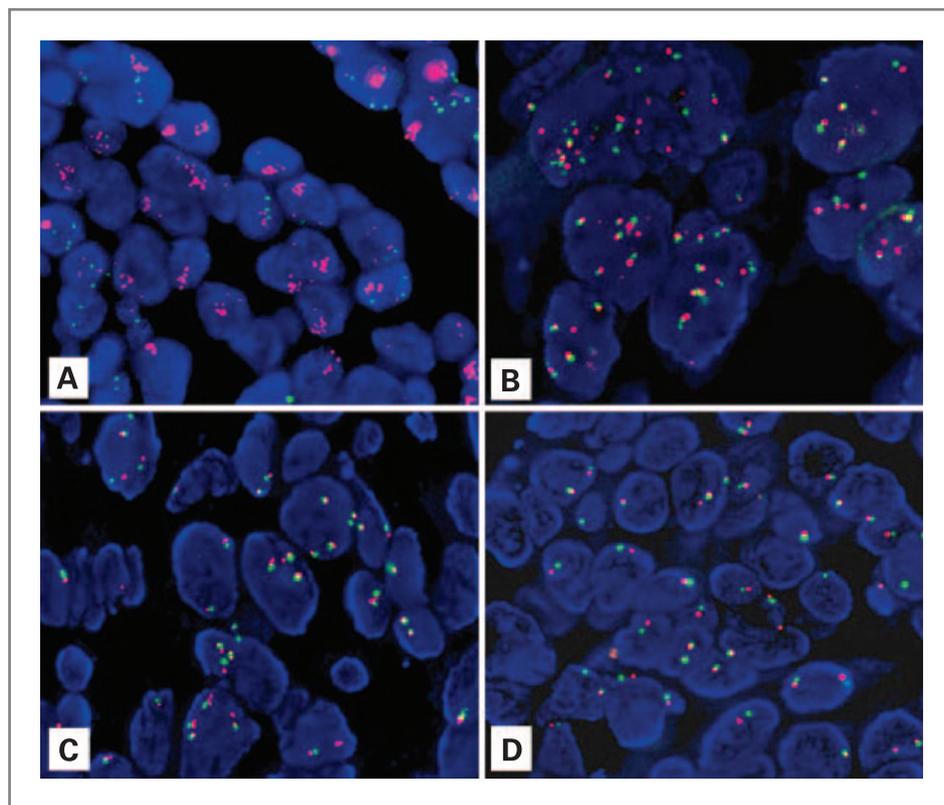
Patient characteristics and *KRAS* status

The baseline and treatment characteristics of the 74 patients are listed in Table 1. Among the patients, 25 (33.8%) achieved an objective tumor response (all partial responses and no complete response), 24 (32.4%) had stable disease, and 25 (33.8%) had progressive disease. Median PFS time was 4.4 months (95% CI, 3.3–5.6), and median OS time was 18.6 months (95% CI, 13.9–23.2). Of the 74 primary tumors analyzed, 20 had *KRAS* mutations (27%). None of the 20 *KRAS* mutation patients had an objective response to cetuximab, whereas 21 of the 54 *KRAS* WT patients were responders (0% versus 38.9%, respectively; $P < 0.001$). Patients with *KRAS* WT had significantly longer OS (25.3 versus 16.0 months; $P = 0.05$) and a significantly longer PFS (5.0 versus 2.5 months; $P = 0.004$) compared with *KRAS* mutation patients.

EGFR FISH analyses

EGFR FISH analysis was successfully detected in 69 of the tumor samples. In the remaining five cases adequate

Fig. 1. Representative FISH detection of *EGFR* copy and chromosome 7 disomy. A, *EGFR* gene focal amplification in tumor cells. B, increase in *EGFR* gene copy number and polysomy of chromosome 7. C, homogenous of *EGFR* gene copy number with a homogeneous chromosome 7 Trisomy. D, no *EGFR* gene copy number and chromosome 7 disomy alteration pattern.



samples for analysis were unavailable due to lack of thin tumor sections of 2 μm provided by a local hospital ($n = 4$) and tissue calcification ($n = 1$). Primary tumor tissues were obtained in 66 cases. There was metastasis in only three cases (tissue samples were obtained from liver, left cervical lymph node, and retroperitoneal lymph node, respectively). Representative patterns of *EGFR* gene signals evaluated by FISH are shown in Fig. 1. Among the 69 patients, 2 (2.9%) had *EGFR* gene amplification in focal areas of the tumor cells, 51 (73.9%) had an *EGFR* FISH pattern of variable ratios of disomy versus polysomy, and 16 (23.2%) had chromosome 7 homogeneous disomy.

We initially analyzed our patients' *EGFR* FISH data according to other scoring systems previously reported in colorectal carcinomas (score B, scores C and D; refs. 9, 11), and in lung carcinoma (score E; refs. 14, 17), as presented in Table 2. When score B was used, 41 patients (59.4%) were *EGFR* FISH positive. *EGFR* FISH-positive patients had a significantly higher RR (39% versus 17.9%; $P = 0.05$) and a trend towards longer OS (18.6 versus 16 months; $P = 0.09$); there was no significant difference in PFS (4.2 versus 3.8 months; $P = 0.69$). Score B showed a 47.9% sensitivity (95% CI, 36.1–59.7) and 76.2% specificity (95% CI, 66.1–86.2). When score C was used, 28 patients (40.6%) were *EGFR* FISH positive. No significant differences in RR (39.3% versus 24.4%; $P = 0.15$), PFS (4.6 versus 3.5 months; $P = 0.97$), and OS (18.9 versus 17.5 months; $P = 0.27$) were observed between *EGFR* FISH-negative and *EGFR* FISH-positive

patients. For this model, sensitivity was 64.6% (95% CI, 53.2–76.0) and specificity was 52.4% (95% CI, 40.6–64.2). When score D was used, 16 patients (23.2%) were classified as *EGFR* FISH positive and no association was detected between *EGFR* FISH positive and clinical outcomes such as RR ($P = 0.34$), PFS ($P = 0.75$), and OS ($P = 0.80$).

We further defined chromosome 7 homogeneous disomy as *EGFR* FISH negative; and gene amplification and pattern of variable ratios of disomy versus polysomy as *EGFR* FISH positive. According to these criteria, 16 (23.2%) cases were classified as *EGFR* FISH negative and 53 (76.8%) as *EGFR* FISH positive. *EGFR* FISH-positive patients had a significantly higher RR (37.7% versus 6.25%; $P = 0.01$) and a trend towards longer PFS (4.5 versus 2.9 months; $P = 0.07$); there was no significant difference in OS (18.6 versus 11.3 months; $P = 0.11$; Table 2 and Fig. 2). This model showed a specificity of 95.2% (95% CI, 90.1–100.3) and a sensitivity of 31.2% (95% CI, 20.3–42.1; Table 2).

Chromosome 7 disomy for prediction with different *KRAS* status

Among the 54 *KRAS* WT patients, 11 (20.4%) had an *EGFR* FISH pattern of chromosome 7 disomy (*EGFR* FISH negative), whereas 37 (68.5%) had variable ratios of disomy versus polysomy, and 2 (3.7%) had gene amplification (*EGFR* FISH positive); the other 4 (7.4%) patients did not have *EGFR* FISH results (Table 3). One of the *EGFR* FISH-negative patients had an objective response

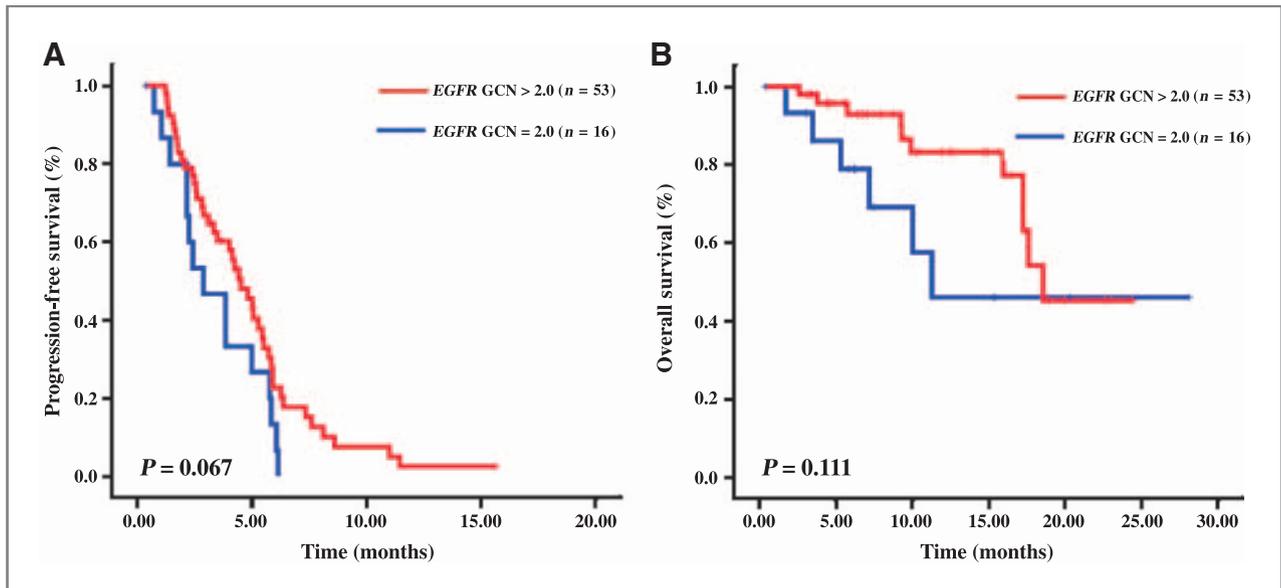


Fig. 2. Kaplan-Meier estimates of progression-free survival (A) and overall survival (B) of in metastatic colorectal cancer patients treated with cetuximab according to *EGFR* GCN patterns evaluated by FISH.

to cetuximab, whereas 20 of the *EGFR* FISH-positive patients were responders (9% versus 51.3%, respectively; $P < 0.01$). Patients who were *EGFR* FISH positive had a significantly longer PFS (5.0 versus 2.3 months; $P = 0.02$) than *EGFR* FISH-negative patients, but there was no significant difference in OS (18.6 versus 10.0 months; $P = 0.16$; Fig. 3A and B). Among the 20 *KRAS* mutation patients, 5 (25%) had an *EGFR* FISH pattern of chromosome 7 disomy (*EGFR* FISH negative), 14 (70.0%) had

variable ratios of disomy versus polysomy, and none had gene amplification (*EGFR* FISH positive); 1 (5.0%) patient did not have an *EGFR* FISH result (Table 3). *KRAS* mutation patients, whether *EGFR* FISH positive or negative, had no objective response to cetuximab. There were no significant differences in RR (0% versus 0%), PFS (2.5 versus 3.8 months; $P = 0.51$), or OS (15.9 versus 11.3 months; $P = 0.43$) between *EGFR* FISH-positive and *EGFR* FISH-negative patients (Fig. 3C and D).

Table 2. Clinical outcomes of the patients according to *EGFR* copy numbers detected by FISH

| | Patients, n (%) | Response, n (%) | PFS (months) | OS (months) | Specificity (95% CI) | Sensitivity (95% CI) |
|--------------------------|-----------------|-----------------|--------------|-------------|----------------------|----------------------|
| EGFR FISH+ (cut point A) | 53 (76.8) | 20 (37.7) | 4.5 | 18.6 | 95.2 (90.1–100.3) | 31.2 (20.3–42.1) |
| EGFR FISH- (cut point A) | 16 (23.2) | 1 (6.25) | 2.9 | 11.3 | | |
| <i>P</i> | | 0.01 | 0.07 | 0.11 | | |
| EGFR FISH+ (cut point B) | 41 (59.4) | 16 (39.0) | 4.2 | 18.6 | 76.2 (66.1–86.2) | 47.9 (36.1–59.7) |
| EGFR FISH- (cut point B) | 28 (40.6) | 5 (17.9) | 3.8 | 16 | | |
| <i>P</i> | | 0.05 | 0.69 | 0.09 | | |
| EGFR FISH+ (cut point C) | 28 (40.6) | 11 (39.3) | 4.6 | 18.9 | 52.4 (40.6–64.2) | 64.6 (53.2–76.0) |
| EGFR FISH- (cut point C) | 41 (59.4) | 10 (24.4) | 3.5 | 17.5 | | |
| <i>P</i> | | 0.15 | 0.97 | 0.27 | | |
| EGFR FISH+ (cut point D) | 16 (23.2) | 6 (37.5) | 5.1 | 18.9 | 28.6 (18.0–39.2) | 79.2 (69.6–88.8) |
| EGFR FISH- (cut point D) | 53 (76.8) | 15 (28.3) | 3.5 | 17.9 | | |
| <i>P</i> | | 0.34 | 0.75 | 0.8 | | |

Cut point A, a mean of ≥ 2.0 *EGFR* gene copy number qualifies the tumor as FISH positive.
 Cut point B, a mean of ≥ 2.47 *EGFR* gene copy number qualifies the tumor as FISH positive.
 Cut point C, a mean of ≥ 2.92 *EGFR* gene copy number qualifies the tumor as FISH positive.
 Cut point D, according to the score system proposed in non-small cell lung cancer, a tumor is defined as FISH positive when $\geq 40\%$ of cells have ≥ 4 copies of *EGFR* or in presence of gene amplification.

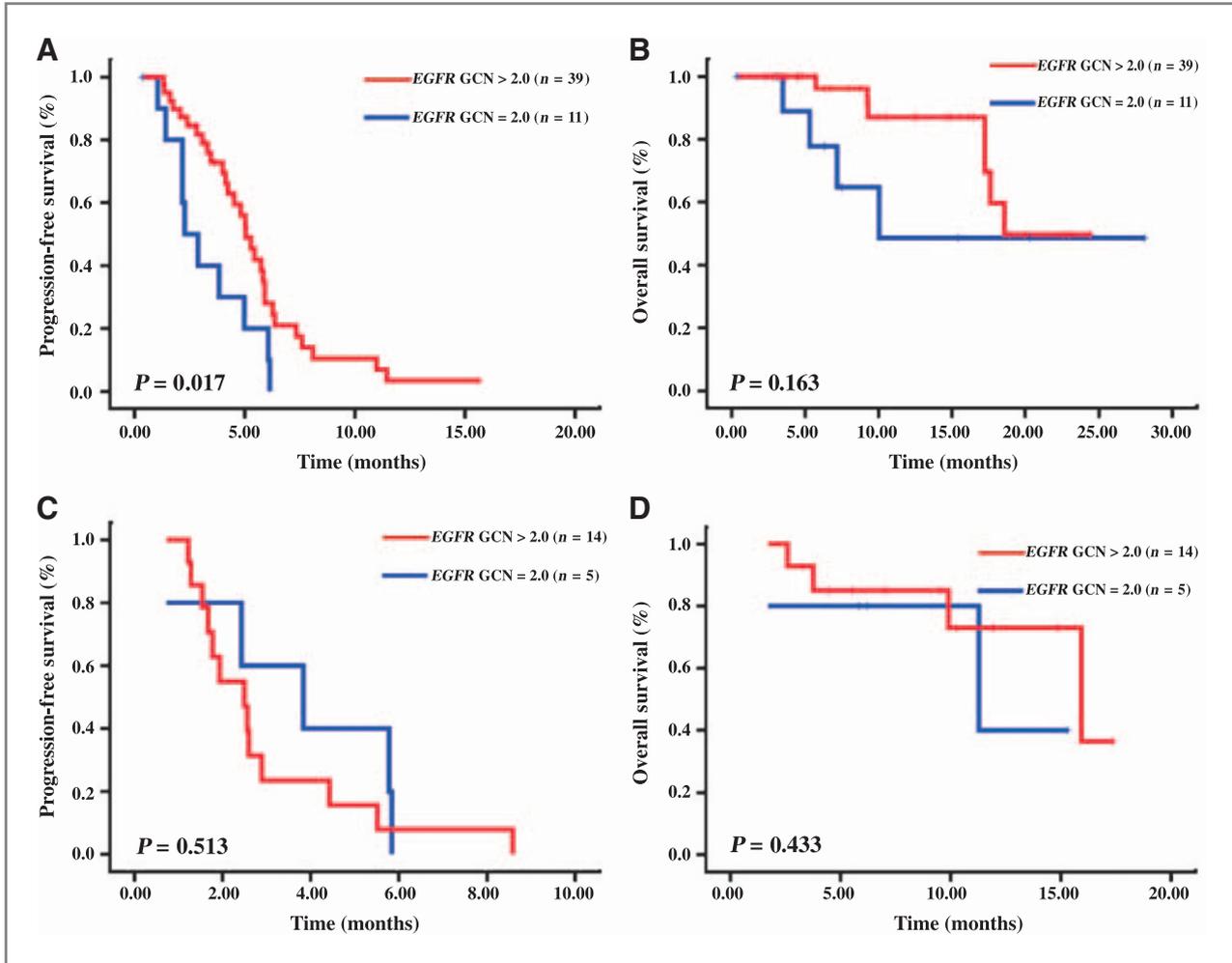


Fig. 3. Kaplan-Meier estimates of progression-free survival (A) and overall survival (B) in *KRAS* WT metastatic colorectal cancer patients and in *KRAS* mutation metastatic colorectal cancer patients (C and D) treated with cetuximab according to *EGFR* GCN patterns evaluated by FISH.

Combination of *KRAS* status and chromosome 7 disomy for prediction

Sixty-nine patients who were successfully tested for *EGFR* by FISH were also tested for *KRAS* mutational status. The

combination of *KRAS* status and *EGFR* FISH patterns were analyzed for response and survival prediction in 41 (55.4%) patients with *KRAS* WT and *EGFR* FISH-positive status and 28 (37.8%) patients with *KRAS* mutation

Table 3. Relationship between tumor response and *KRAS* status combined with *EGFR* GCN

| | <i>KRAS</i> wild-type (n = 54)* | | <i>KRAS</i> mutation (n = 20)† | |
|---------------------|----------------------------------|------------------------------|--------------------------------|------------------------------|
| | <i>EGFR</i> GCN No. = 2.0 No. | <i>EGFR</i> GCN > 2.0 No. | <i>EGFR</i> GCN = 2.0 No. | <i>EGFR</i> GCN > 2.0 No. |
| Response (CR+PR) | 1 | 20 | 0 | 0 |
| Nonresponse (SD+PD) | 10 | 19 | 5 | 14 |

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

*Fifty metastatic colorectal cancer patients got the result of *EGFR* GCN by FISH.

†Nineteen metastatic colorectal cancer patients got the result of *EGFR* GCN by FISH.

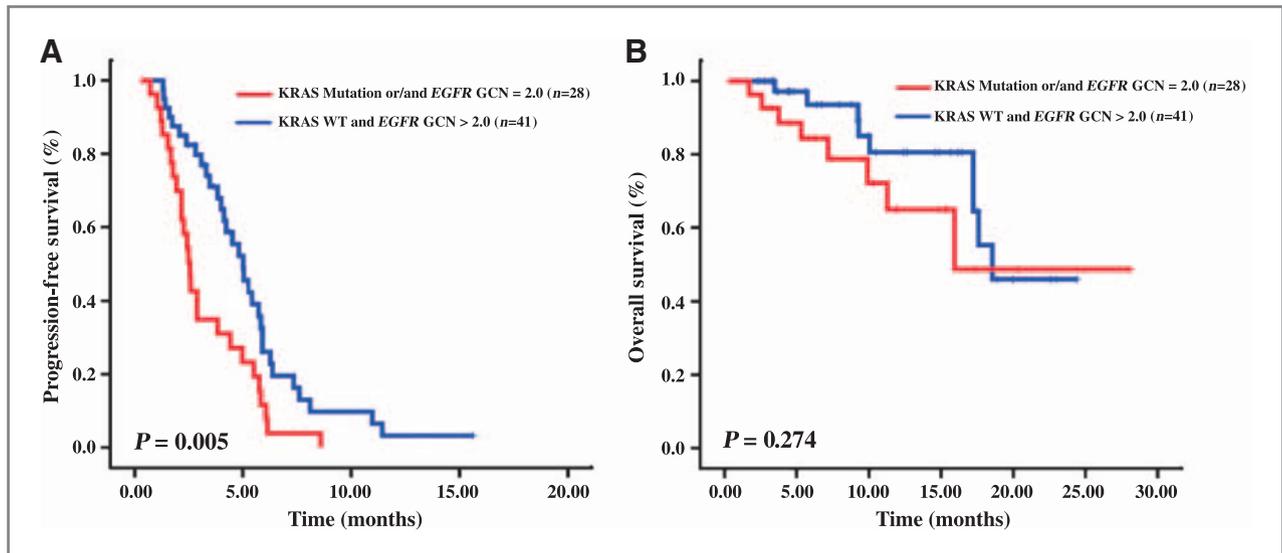


Fig. 4. Kaplan-Meier estimates of progression-free survival (A) and overall survival (B) in metastatic colorectal cancer patients treated with cetuximab according to the combination of *KRAS* status and *EGFR* GCN patterns.

and/or *EGFR* FISH-negative status. One of the 28 *KRAS* mutation and/or *EGFR* FISH-negative patients had an objective response to cetuximab, whereas 20 of 41 *KRAS* WT and *EGFR* FISH-positive patients were responders (3.6% versus 48.8%, respectively; $P < 0.001$). Patients with *KRAS* WT and *EGFR* FISH-positive status had a significantly longer PFS (5.0 versus 2.6 months; $P = 0.005$) than *KRAS* mutation and or *EGFR* FISH-negative patients, and a trend towards longer OS (18.6 versus 16.0 months; $P = 0.27$) (Fig. 4). This model showed a sensitivity of 65.2% (95% CI, 51.7–78.7) and a specificity of 100% (95% CI, 100–100).

Discussion and Conclusion

Although recent studies have confirmed that *EGFR* GCN assessed by FISH can influence the response to anti-*EGFR* mAb therapy in metastatic colorectal cancer, methods of tissue processing and *EGFR* scoring systems were not standardized among these studies (8–11). In our present study, we assessed the value of an *EGFR* GCN cutoff point according to what has been previously reported in colorectal carcinomas and in lung carcinomas (9–11, 14–17). Our results agree with other studies that the cutoff point defined in lung carcinomas is not suitable for predicting the response to cetuximab in metastatic colorectal cancer. For metastatic colorectal cancer patients, the nonincreased *EGFR* GCN status rather than the increased is the most accurate predictive factor for clinical outcome, so effort should be made to better define the low copy number pattern. In our study, using the cutoff of ≥ 2.92 *EGFR* GCN as FISH positive as proposed by Cappuzzo (11), there was no difference in RR PFS, or OS between the negative and positive groups. Using the cutoff of ≥ 2.47 *EGFR* GCN as positive as proposed by Sartore-Bianchi (9), positive patients had a significantly higher RR (39% versus

17.9%; $P = 0.05$) and a trend towards longer OS (18.6 versus 16 months; $P = 0.09$), suggesting that the cutoff point of 2.47 *EGFR* GCN seems more suitable for our study.

The thickness of tumor sections may influence the judgment and definition of *EGFR* GCN. Our study evaluated a large number of cells in thin sections of 2 μm to avoid overlapping of nuclei, which was the same procedure as in Sartore-Bianchi's study (9), whereas Cappuzzo's (11) analysis was conducted using 4- μm tissue sections. The thinner tumor sections could be responsible for the lower cutoff point associated with clinical outcome reported in different studies.

However, it is difficult to determine exactly whether the *EGFR* GCN is more or less than 2.47/nucleus in clinical practice. From a morphologic point of view, chromosome 7 disomy is easier to identify and assess than an increase in *EGFR* GCN. Sartore-Bianchi et al. have reported that most metastatic colorectal cancer patients with nonincreased *EGFR* GCN displayed homogeneous disomy (9). There are no data, however, to support chromosome 7 disomy being used in clinical practice at this time. Our present study revealed that only 1 of the 16 (6.2%) disomy patients responded to treatment with cetuximab. The specificity of prediction for nonresponsive patients was 95.2%, but the sensitivity of prediction for responsive patients was quite low (31.2%). A biomarker that definitively predicts a negative response is as useful as one that predicts a positive response. Using chromosome 7 disomy as criteria, our study indicated that 23% of metastatic colorectal cancer patients could be excluded from unnecessary treatment with cetuximab. Sartore-Bianchi et al. have reported that none of the 38 patients with a mean *EGFR* GCN of < 2.47 /nucleus responded to panitumumab whereas 6 of 20 patients with a mean *EGFR* GCN of > 2.47 /nucleus achieved a response (9). If their data are analyzed using

chromosome 7 disomy as criteria, the specificity of prediction for nonresponsive patients is still 100%, but the sensitivity of prediction for responsive patients is lower than they reported. We agree with Mauro Moroni's opinion that from a clinical point of view, we can risk treating a nonresponsive patient, but we cannot risk not treating a potentially responsive patient (12). Furthermore, the most important point is that chromosome 7 disomy is easier to detect than an increase in *EGFR* copy number, and therefore, might enable a more reproducible FISH assay in clinical practice.

Using the cutoff value of chromosome 7 disomy, our data showed a trend towards longer PFS (4.5 versus 2.9 months), but the difference did not achieve statistical significance. However, a subgroup of *KRAS* WT patients who were *EGFR* FISH positive had a significantly longer PFS (5.0 versus 2.3 months; $P = 0.02$) than *EGFR* FISH-negative patients, whereas in *KRAS* mutant patients, no matter whether they were *EGFR* FISH positive or negative, there were no significant differences in RR (0% versus 0%), PFS (2.5 versus 3.8 months; $P = 0.51$), or OS (15.9 versus 11.3 months; $P = 0.43$). Our study supported the hypothesis that in the presence of the *KRAS* gene wild-type, tumor growth is probably mainly driven by the *EGFR* pathway and this biological characteristic is evoked by an increase in *EGFR* copy number. In the presence of *KRAS* mutation, however, *EGFR* signaling transduction gets rid of the control of the upstream receptor and resistance to anti-*EGFR* treatments.

Furthermore, our study showed that the combined detection of *EGFR* GCN with *KRAS* mutations provided better predictive values for selecting metastatic colorectal cancer patients who would respond to cetuximab, and especially with regard to identifying those patients with tumors which are either *EGFR* FISH negative or *KRAS* mutant-type status and are unlikely to benefit from anti-*EGFR* mAb therapy. To the best of our knowledge, there has been only one previous report, that from Personeni et al. (10), that described that the relationship between mean *EGFR* GCN and survival differs between wild-type and mutant patients, but there was no assessment of the value of

combination detection of the two markers for selection of patients for anti-*EGFR* therapies.

Despite the high negative predictive value of both *EGFR* FISH and *KRAS* mutation status in our cohort, the positive predict value was still low, there was still a significant percentage of patients with an increased gene copy number and *KRAS* WT status who were nonresponsive. Therefore, further identification and combination evaluation of other predictive biomarkers, e.g., *EGFR* downstream genes such as *BRAF*, *PIK3C2A*, and *PTEN*, is imperative to improve the selection of candidates for mAb treatment. Due to the retrospective nature and limited number of cases of this study, a prospective, large sample and multicenter study on *KRAS* mutation status and *EGFR* GCN and their predictive value for selecting individual metastatic colorectal cancer patients who would respond to cetuximab is further needed to validate our findings.

In conclusion, the results of the current study show firstly that it may be feasible to consider *EGFR* FISH pattern of chromosome 7 disomy as a negative predictive factor for cetuximab response in *KRAS* WT metastatic colorectal cancer. Together with *KRAS* mutation, chromosome 7 disomy predicts metastatic colorectal cancer patients will not respond to cetuximab.

Disclosure of Potential Conflicts of Interest

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