Cancer Therapy: Clinical

Clinical Usefulness of *EGFR* Gene Copy Number as a Predictive Marker in Colorectal Cancer Patients Treated with Cetuximab: A Fluorescent *In situ* Hybridization Study

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Abstract

Purpose: To evaluate the usefulness and the pitfalls inherent to the assessment of the epidermal growth factor receptor (*EGFR*) gene copy number (GCN) by fluorescence *in situ* hybridization (FISH) for outcome prediction to cetuximab in metastatic colorectal cancer. The value of testing *KRAS* mutation status, in addition to *EGFR* GCN, was also explored.

Experimental Design: FISH analysis of 87 metastatic colorectal cancer patients treated with cetuximab was done, recording individual GCN per cell and using different samples per tumor. Performances of published cutoff points and different summaries of *EGFR* GCN distribution were assessed for response prediction.

Results: In our data set, two published cutoff points performed less well than in their training set, yielding positive predictive values and negative predictive values between 40.0% and 48.3% and between 81.0% and 86.5%, respectively. Among summaries of GCN distribution explored, mean and right-tailed distribution of GCN yielded the highest performances. A mean *EGFR* GCN ≥ 2.83 provided an area under the curve of 0.71. Important heterogeneity of repeated measures of mean *EGFR* GCN was observed within tumors (intraclass correlation, 0.61; within-class SD, 0.40), leading to potential misclassifications of FISH status in 7 of 18 (38.8%) patients if a cutoff point were used. In multivariable analysis, *EGFR* GCN testing provided significant information independent of the *KRAS* status to predict response ($P = 0.016$) and overall survival ($P = 0.005$).

Conclusions: We confirm the association between increased *EGFR* GCN and outcome after cetuximab. However, because of reproducibility concerns, any decision making based on published cutoff points is not warranted.

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Novel strategies that target the epidermal growth factor receptor (*EGFR*) have led to the clinical development of a variety of agents, including small inhibitory molecules and monoclonal antibodies (mAb). Two such mAbs, panitumumab and cetuximab, are active in metastatic colorectal cancer, but only subgroups of patients respond to these agents (1–3) and reliable markers predictive of treatment benefit still need to be defined.

Variations of gene copy numbers (GCN), either in terms of gains or losses, reflect the many different routes taken by individual tumors to disrupt/escape mechanisms governing normal cellular behavior. These genomic aberrations have been successfully investigated by fluorescence *in situ* hybridization (FISH) in a number of malignancies. Importantly, three recently published series have reported on enhanced sensitivity to anti-*EGFR* mAbs in colorectal cancer patients harboring an increase of mean *EGFR* GCN by FISH (4–6). In most solid tumors, including non–small-cell lung cancer and colorectal cancer, the best characterized mechanisms underlying increased *EGFR* GCN are gene amplification and chromosome 7 polysomy (4–7). Generally, amplification is representative of high-level

genomic gain and it is readily identifiable by FISH. Conversely, polysomy mirrors variable degrees of chromosomal gains, making the evaluation of subtle changes of GCN somehow more arbitrary. Historically, the retrieval of relevant cutoff points for gene amplification and chromosome polysomy to predict outcome after targeted therapies represents a major challenge. In the field of breast cancer, this is well reflected by the debate still ongoing over the classification of *HER2*-positive tumors to predict trastuzumab efficacy by FISH analysis (8).

In addition, evidence from earlier studies on DNA ploidy and corresponding variations of chromosome copy number (9) indicates that copy number changes in most colorectal cancer tumors might be very heterogeneous, thereby increasing the difficulties encountered while assessing *EGFR* GCN. However, despite the implications of these features for the global interpretation and the reproducibility of the assay in colorectal cancer, to our knowledge, no study thus far has addressed this critical point with respect to outcome prediction after anti-EGFR mAbs.

The aim of the present study was to evaluate the clinical usefulness of *EGFR* GCN by FISH in predicting outcome to cetuximab. To this end, we first evaluated whether previously generated cutoff points (5, 6) could be validated in our independent series. Second, in view of the important intrapatient and interpatient variability of *EGFR* GCN, we assessed optimized ways of capturing and reporting individual observations and repeated the process of defining an optimal cutoff point on this data set. Furthermore, using a multivariable analysis, we explored the combined use of *EGFR* GCN with the *KRAS* mutation status of the tumor, currently one of the best-established markers for outcome prediction after cetuximab in colorectal cancer (10–12). Finally, we also explored the value of *HER2* GCN testing, alone or in combination with *EGFR* GCN.

Materials and Methods

Patients and data collection. We assessed irinotecan-refractory patients treated at four Belgian Institutions, in the setting of the following trials of cetuximab: BOND (1), SALVAGE (13), Babel, and EVEREST (14). Cetuximab was administrated as a third or subsequent line of treatment for advanced disease, alone or in combination with irinotecan. Excepting the EVEREST trial (14), where patients could receive escalating doses of cetuximab up to 500 mg/m^2, all patients were treated with cetuximab at a standard loading dose of 400 mg/m^2, followed by weekly infusions of 250 mg/m^2. Irinotecan was administered according to doses and schedules in use before the onset of irinotecan resistance. Treatment was continued until progression of disease or toxicity occurred. Objective response was evaluated every 6 wk by computed tomography scan according to the Response Evaluation Criteria in Solid Tumors. Patient inclusion in the current study was based on the availability of sufficient formalin-fixed paraffin-embedded pretreatment tumor tissue. This study was approved by the Institutional Review Board.

EGFR and HER2 by FISH. Serial sections (4 μm) were prepared from formalin-fixed paraffin-embedded tissue. The presence of invasive carcinoma was confirmed by a pathologist on a sequential slide stained with H&E. *EGFR* and *HER2* GCN were investigated using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen and LSI HER-2/neu SpectrumOrange/CEP 17 SpectrumGreen probes (Vysis), respectively. Following deparaffinization and dehydration, pretreatment and enzyme digestion were done using the SPoT-Light Tissue Pretreatment Kit

(Zymed) according to the manufacturer's recommendations. Probes were applied to target area and codenatured at 85°C for 5 min before overnight hybridization at 37°C. Posthybridization washes were done in 0.4× SSC/0.3% NP40 at 73°C for 2 min and in 2× SSC/0.1% NP40 for 1 min. Slides were counterstained with 4′,6′-diamidino-2-phenyl-indole and were stored at -20°C before evaluation. Captures ere acquired using a Zeiss Axioplan 2 microscope (Zeiss) equipped with a charge-coupled device camera and a MetaSystem Isis software (MetaSystem). Sequential focus stacks with 0.8-μm intervals were merged into a single image to reduce thickness-related artifacts.

FISH interpretation. Probe signals were enumerated in individual nuclei if they were bright, distinct, and easily assessable against a dark background relatively free of fluorescent particles and haziness. For both *EGFR* and *HER2*, the number of hybridization signals representing genes and chromosome centromeres was determined observing four to five tumor areas selected in proximity of relevant histologic features identifiable on the H&E slide. At least 20 representative nuclei, distributed in two to three microscope contiguous fields within an individual tumor area, were selected for scoring. The number of gene copies and the number of centromeres per nucleus were individually recorded for at least 100 nuclei. Chromosome polysomy was defined as ≥3 gene signals per nucleus paralleled by similar increases in chromosome centromere signals. *EGFR* and *HER2* amplifications were defined according to cutoff points previously reported in non–small-cell lung cancer (15, 16).

EGFR and HER2 by immunohistochemistry. *EGFR* and *HER2* protein expression was detected by immunohistochemistry using respectively the EGFR PharmDx kit and the Dako HercepTest (DakoCytomation) and in accordance to methods described elsewhere (17, 18). Protein expression was reported as membranous brown staining of neoplastic cells using a three-tier system ranging from 1+ (weak intensity; faint brown membranous staining) to 3+ (strong intensity; dark brown or black membranous staining producing a thick outline, complete or incomplete of the neoplastic cell). Tumors were deemed *EGFR* positive when ≥1% of the tumor cells exhibited membranous staining of any intensity. HER2 protein expression was deemed positive with intensity scores of 2+ or 3+ in ≥10% tumor cells.

KRAS mutational analysis. Mutational analysis of exon 2 (codons 12 and 13) was done as previously reported (11). Briefly, the presence of *KRAS* mutations was determined by an allelic discrimination assay on a 7500HT Real Time PCR System (Applied Biosystems).

Table 1. Baseline demographic characteristics of the patients who underwent FISH for *EGFR*, alone and in combination with *HER2*, and *KRAS* mutational analysis

Characteristic	EGFR by FISH	EGFR and HER2 by FISH
	No. patients (%)	
Sex		
Male	49 (56)	37 (58)
Female	38 (44)	27 (42)
Age, y		
Median	58.6	56.2
Range	25.7-80.1	25.7-80.1
Cetuximab doses (mg/m^2)		
250	75 (86)	55 (86)
≥250	12 (14)	9 (14)
Treatment		
Cetuximab monotherapy	18 (21)	12 (19)
Cetuximab and irinotecan	69 (79)	52 (81)
KRAS mutation status		
Wild-type	58 (67)	44 (69)
Mutant	29 (33)	20 (31)

Table 2. Diagnostic indices obtained when applying previously published cutoff points to the current data set

	Score A (95% CI)	Score B (95% CI)
Sensitivity	80.0 (59.3-93.2)	56.0 (34.9-75.6)
Specificity	51.6 (38.6-64.5)	75.8 (63.4-85.8)
PPV	40.0 (26.4-54.8)	48.3 (29.5-67.5)
NPV	86.5 (71.2-95.5)	81.0 (68.6-90.1)
Accuracy	59.8 (48.7-70.2)	70.1 (59.4-79.5)
c-index	0.66	0.66

NOTE: Score A: Sartore-Bianchi et al. (5); cutoff point, 2.47. Score B: Cappuzzo et al. (6); cutoff point, 2.92.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

Statistical analysis. The distribution of gene copies per nucleus was summarized using various indices of location and variability, including the mean, median, SD, maximum GCN in an individual cell, and a set of percentiles representing the right tail of the distribution. To verify which of these summary statistics (called indices in the remainder) was most informative to predict response, a separate logistic regression model was constructed for each of them. The presence of amplification was added as a binary predictor. Nonlinear relations were allowed using restricted cubic splines (19). For each model, the performance was quantified by the *c*-index, which represents the area under the curve, with values ranging between 0.5 (fortuitous prediction) and 1 (perfect discrimination). Likelihood ratio tests were done to verify whether the combinations of different indices significantly provide supplementary information. Cutoff points were determined by means of the receiver operating characteristics (ROC) curve analysis, and a nonparametric bootstrap procedure was followed to construct a 95% confidence interval (95% CI). Exact 95% CIs were constructed for diagnostic indices including sensitivity, specificity, positive predictive value, and negative predictive value. When two or more samples were available per patient, data from only one sample were randomly retained in the analysis. Spearman correlations were calculated to explore the relation between continuous variables. Using the term "class" to indicate tumor samples and patients, intraclass correlations and corresponding within-class SDs were calculated to quantify the within-tumor and within-patient heterogeneities observed in repeated measures of the mean *EGFR* GCN. Briefly, the intraclass correlation represents the ratio of the variance between the classes (for instance, the variance between tumor samples) and the total variance. The latter is the sum of the between-class variance and within-class variance (e.g., the variance of the tumor areas within an individual sample). Progression-free survival was defined as the time between the first day of cetuximab treatment until either progression of disease, death from any cause, or last radiologic assessment. Overall survival was defined as the period from start of cetuximab until death. Kaplan-Meier plots were constructed for progression-free survival and overall survival and compared between groups using the log-rank test. A logistic regression model for objective response and a Cox regression model for survival including the interaction between mean *EGFR* GCN and *KRAS* mutation status were used. All analyses were done using the statistical package SAS (version 9.1.3). *P* < 0.05 was considered significant.

Results

Patient population

Overall, 96 patients were evaluated, of which 87 successfully underwent *EGFR* testing on 112 samples whereas 76 underwent *HER2* testing on 88 samples. Concomitant *EGFR* and *HER2* testing was done in 64 patients. Their baseline and treatment characteristics are summarized in Table 1. Prevalent tumor histology was adenocarcinoma not otherwise specified (95%) whereas other histologic types were classified as mucinous (4%) or adenosquamous carcinoma (1%). For patients treated with cetuximab alone or in combination with chemotherapy, objective response rates were 16.7% (3 of 18) and 31.9% (22 of 69), median progression-free survival were 3.5 (95% CI, 2.8-5.5) and 5.5 (95% CI, 3-6.9) months, and median overall survival were 15 (95% CI, 12-24) and 21 (95% CI, 12-30) months, respectively.

EGFR gene copy number and protein expression

EGFR expression by immunohistochemistry was determined in 72 samples; 65 samples were deemed *EGFR* positive and 7 were *EGFR* negative. There was no significant correlation between *EGFR* staining intensity and any level of mean *EGFR* GCN considered (Spearman correlation = -0.21; *P* = 0.26), with the exception of four samples (originating from two patients) displaying *EGFR* amplification, all of which also exhibited a strong staining (3+) by immunohistochemistry. Interestingly, in one of these four samples, immunohistochemical staining showed regional patterns with strong intensity, perfectly overlapping with well-delimited tumor areas of gene amplification on a sequential FISH slide. In contrast, surrounding regions showing polysomy, instead of amplification, exhibited only moderate staining at the protein level. Furthermore, in one patient, we observed that *EGFR* amplification observed in the primary tumor was conserved in the corresponding liver metastasis.

Testing the performance of previously published approaches for response prediction

When tested in the current independent series, two cutoff points based on mean GCN previously published (5, 6)

Table 3. Median progression-free survival and overall survival obtained when applying previously published cutoff points to the current data set

Score		Progression-free survival (mo)		Overall survival (mo)	
		Median (95% CI)	*P* (log-rank)	Median (95% CI)	*P* (log-rank)
Score A	<Cutoff point	3.9 (2.8-5.5)	0.34	8.4 (5.3-14.2)	0.34
	≥Cutoff point	5.5 (3.7-6.9)		9.1 (6.9-12.8)	
Score B	<Cutoff point	4.3 (2.8-5.5)	0.42	8.5 (5.7-12.6)	0.23
	≥Cutoff point	5.5 (2.8-8.3)		9.6 (6.2-13.7)	

NOTE: Score A: Sartore-Bianchi et al. (5); cutoff point, 2.47. Score B: Cappuzzo et al. (6); cutoff point, 2.92.
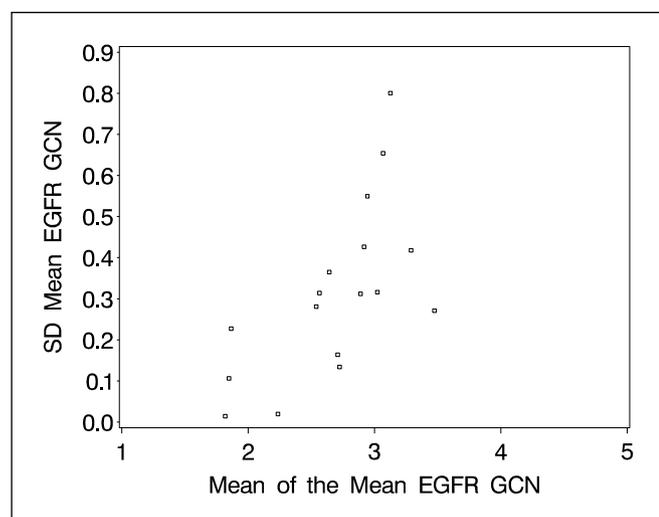
Fig. 1. Visualization of the relation between the mean of the mean *EGFR* gene copy number and the SD obtained in patients with multiple tumor samples originating either from the same or different tumor sites.

provided the performance indices reported in Table 2 for response prediction. Moreover, when applied to our series, neither cutoff point significantly predicted outcome in terms of progression-free survival and overall survival (Table 3).

EGFR gene copy number heterogeneity within a sample and within a patient

(a) Within-sample heterogeneity. To assess the within-sample heterogeneity, we considered the variability of mean *EGFR* GCN obtained in multiple tumor areas from individual tumor samples. To this end, we used 27 slides submitted to FISH analysis, where two to five tumor areas had been assessed separately, each with a number of counted cells ranging from 19 to 50 (median, 20.5). Overall, data on 120 different tumor areas were available (Supplementary Table S4). A relevant heterogeneity of mean *EGFR* GCN among different tumor areas belonging to the same slide was observed. The intraclass correlation equaled 0.76, whereas the within-sample SD of the mean *EGFR* GCN equaled 0.29. In particular, this latter finding implies that 95% of the mean *EGFR* GCN values obtained from various tumor areas will cover the range (-0.58; +0.58) around the true mean *EGFR* GCN of the tumor sample.

(b) Within-patient heterogeneity. To assess the within-patient heterogeneity, we considered the variability of mean *EGFR* GCN obtained in multiple samples belonging to the same tumor. To this end, we considered a set of 17 patients, among which 6 had three samples available and 11 had two samples. Multiple samples included in this analysis originated either from the same site or from different sites (Supplementary Table S5). Mean *EGFR* GCN were based on 100 nuclei counted on slides from each sample considered. The intraclass correlation equaled 0.61, whereas the within-patient SD of the mean *EGFR* GCN equaled 0.40. This implies that 95% of the mean *EGFR* GCN values obtained from multiple samples of the same tumor will cover the range (-0.80; +0.80) around the true mean *EGFR* GCN of the tumor.

In addition, we hypothesized that the within-patient heterogeneity might be inflated because the multiple samples

considered could originate from the primary tumor, from metastases, or both. Therefore, should there be a systematic difference in mean *EGFR* GCN between primary tumors and related metastatic lesions, then part of the reported within-patient SD originates from this difference. However, after correcting for a possible systematic difference, the recalculated intraclass correlation and the within-patient SD did not change and were equal to 0.61 and 0.40, respectively. Note that a Mann-Whitney *U* test confirmed that there is no evidence to claim a difference between primary or metastatic tumor samples in terms of mean *EGFR* GCN ($P = 0.97$).

Lastly, we found that, independently from the origin of an individual tumor block, the within-patient variability depends on the mean *EGFR* GCN level (Spearman correlation = 0.65; $P = 0.005$; Fig. 1).

Exploring alternatives of reporting *EGFR* gene copy number for response prediction

Given the relevant heterogeneity of *EGFR* GCN, we reasoned that summaries of the GCN distribution other than the mean could be predictive for response. To test the hypothesis that increases of *EGFR* GCN in a limited subset of cells would be more predictive of response, we explored the diagnostic performance of the decision rule "the proportion of cells, having a GCN higher than a specific value r, being higher than some value s." In other words, this rule allows to analyze the predictive value of specific increases of GCN within subsets of polysomic cells. However, among all possible combinations of cutoff points r and s explored, the highest c-index reached was equal to 0.71, which did not outperform the use of the mean *EGFR* GCN (Supplementary Table S6).

The mean *EGFR* GCN and the statistics summarizing the right tail of the *EGFR* GCN distribution (i.e., the 95th percentile and the highest GCN in an individual cell) eventually contained the highest amount of information to predict response. In fact, the c-index equaled 0.738 and 0.732 for the mean and the 95th percentile *EGFR* GCN, respectively. Allowing a more flexible (i.e., nonlinear) relation between the
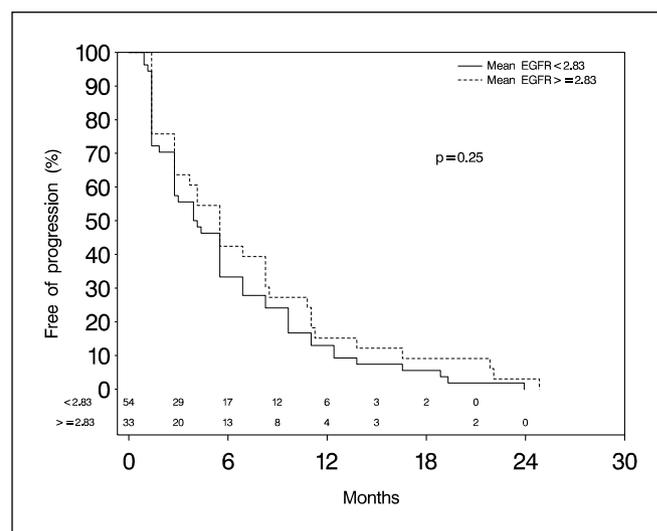
Fig. 2. Progression-free survival in *EGFR* FISH – positive and *EGFR* FISH – negative patients, according to the cutoff point of 2.83 mean GCN identified by the ROC analysis.
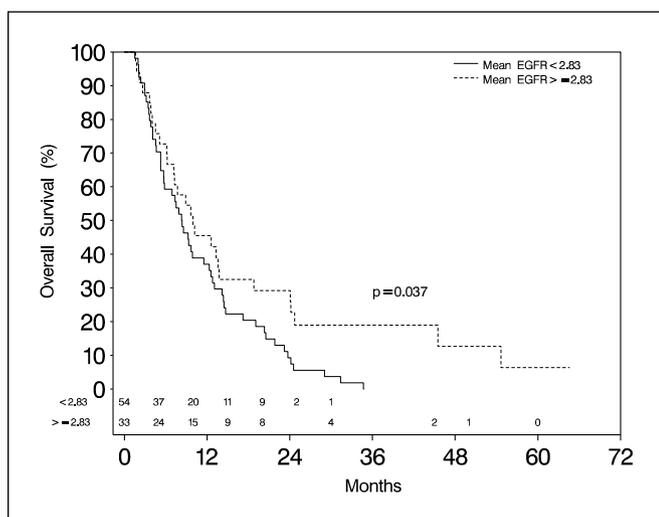
Fig. 3. Overall survival in *EGFR* FISH – positive and *EGFR* FISH – negative patients, according to the cutoff point of 2.83 mean GCN identified by the ROC analysis.

summary statistic and outcome did not substantially increase the *c*-index. Note that compared with the mean GCN, the highest GCN in an individual cell and the 95th percentile represent less attractive parameters to evaluate because their values are dependent on the number of cells counted, whereas the mean GCN is not. Given these reasons, we elected to use only the mean GCN for further outcome prediction.

Defining a cutoff point for mean *EGFR* gene copy number to predict response

Using ROC curve analysis, a mean *EGFR* GCN ≥2.83 (95% CI, 2.26-3.19) was found to be the most suitable cutoff point to discriminate between responders and nonresponders (*c*-index = 0.71). According to this cutoff point, sensitivity was equal to 17 of 25 (68%; 95% CI, 46.5-85.1%), specificity to 46 of 62 (74.2%; 95% CI, 61.5-84.5%), positive predictive value to 17 of 33 (51.5%; 95% CI, 33.5-69.2%), negative predictive value to 46 of 54 (85.2%; 95% CI, 72.9-93.4%), and overall accuracy was 63 of 87 (72.4%; 95% CI, 61.8-81.5%). Similar performances were observed using the most suitable cutoff point that represents the percentage of cells displaying chromosome 7 polysomy, found to be equal to 56% (Supplementary Table S7). Due to the strong correlation existing between the mean *EGFR* GCN and the 95th percentile GCN (Spearman ρ = 0.85; P < 0.0001), or the percentage of cells exhibiting chromosome 7 polysomy (Spearman ρ = 0.85; P < 0.0001), the combination of these sources of information to improve response prediction did not turn out to be meaningful.

According to the ROC-based cutoff point that was generated to predict response, longer progression-free survival (median, 5.5 versus 4.0 months; P = 0.25; Fig. 2) and overall survival (median, 10 versus 8.3 months; P = 0.037; Fig. 3) were observed in patients with high mean GCN. Note that due to the within-patient heterogeneity of the mean *EGFR* GCN, the use of a cutoff point can easily lead to another classification (FISH+ or FISH-) of a patient depending on the sample used. In fact, in 7 of 18 (38%) patients having at least two tumor samples available, we observed a discrepancy in the obtained classification (Fig. 4).

### HER2 gene amplification and protein expression

HER2 amplification was detected in samples from 4 of 76 (5.2%) patients tested; 3 of them, who were concomitantly tested by immunohistochemistry, displayed a positive *HER2* staining. Conversely, immunohistochemical staining was negative in 35 patients, none of which exhibited *HER2* amplification.

HER2 gene copy number for response prediction

With respect to various indicators of *HER2* GCN, similar analyses for response prediction were made as for *EGFR* GCN (data not shown). Mean *HER2* GCN taken as a single predictor yielded only a very weak *c*-index of 0.617. Interestingly, in four patients with *HER2* amplification, the mean *EGFR* GCN was ≥2.83, but no response was observed on cetuximab-based therapy. Because *EGFR* and *HER2* mean GCN were found to be only partially related (R^2 = 0.21, Pearson correlation squared), we reasoned that coupling *EGFR* and *HER2* information might improve the predictive value of the assessment. Adding to mean *EGFR* GCN and *EGFR* amplification the combined information on mean *HER2* GCN and *HER2* amplification significantly increased the *c*-index (0.746; P = 0.036). Nevertheless, this increase was largely due to *HER2* amplification, but not to *HER2* gains deriving from chromosome 17 polysomy. This is reflected by the fact that only the addition of the *HER2* amplification, but not mean *HER2* GCN, yielded a significant increase of the *c*-index (P = 0.018).

Combination of *EGFR* gene copy number and *KRAS* mutational status for response and survival prediction

All 87 patients successfully tested for *EGFR* by FISH were also tested for *KRAS* mutational status. The median mean *EGFR* GCN was 2.26 in *KRAS*-mutant tumors and 2.77 in tumors with a *KRAS*-wild-type status (Mann-Whitney *U* test, P = 0.022). In contrast, no significant difference was observed in *HER2* mean GCN according to *KRAS* status (P = 0.15). None of 29 (33%) mutant patients responded; hence, no effect of mean *EGFR* GCN could be observed. Conversely, among
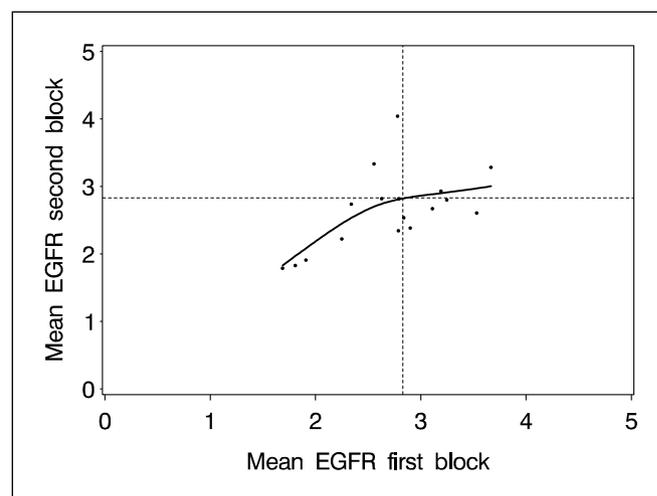
Fig. 4. Classification of the FISH status according to the 2.83 mean *EGFR* gene copy number cutoff point in multiple samples belonging to the same tumor. The vertical and horizontal dashed lines are representative of the 2.83 mean GCN cutoff point identified by the ROC analysis.

58 (67%) *KRAS*-wild-type patients, 63% (17 of 27) of those with a mean *EGFR* GCN ≥2.83 responded, compared with 26% (8 of 31) patients with a mean *EGFR* GCN <2.83. ROC analysis on the 58 *KRAS*-wild-type patients indicated a cutoff point of 2.76 mean *EGFR* GCN, with sensitivity equal to 72.0% (95% CI, 50.6-87.9%), specificity to 66.6% (95% CI, 48.1-82.0%), positive predictive value to 62.0% (95% CI, 42.2-79.3%), negative predictive value to 75.8% (95% CI, 56.4-89.7%), and the *c*-index equal to 0.69.

A logistic regression model including *KRAS* mutation status, dichotomized *EGFR* GCN, and their interaction indicated that, once *KRAS* status is known, the mean *EGFR* GCN still contributes information for the prediction of objective response ($P = 0.016$). In a Cox regression model including the interaction between mean *EGFR* GCN and *KRAS* status, mean *EGFR* GCN still contributes information also for overall survival ($P = 0.005$), but not for progression-free survival ($P = 0.14$). An interaction between *EGFR* GCN and *KRAS* mutation status was observed for overall survival ($P = 0.002$) and progression-free survival ($P = 0.056$), indicating that the relation between mean *EGFR* GCN and survival differs between wild-type and mutant patients. In fact, in *KRAS*-wild-type patients, a FISH-positive status was related with longer median overall survival (13.3 versus 8.4 months; $P = 0.019$) and a nonsignificant increase in progression-free survival (6.9 versus 4.4 months; $P = 0.16$). However, in *KRAS*-mutant patients, a mean *EGFR* GCN ≥2.83 was related with shorter median progression-free survival (2.8 versus 3.9 months; $P = 0.084$) and overall survival (3.8 versus 7.5 months; $P = 0.006$).

Discussion

In this study, the primary aim was to explore the use of the FISH assay in predicting outcome after cetuximab-based therapy for metastatic colorectal cancer. Testing the performance of specific cutoff points of mean *EGFR* GCN previously reported (5, 6), we found that the implementation of this marker in clinical practice is challenging. Nevertheless, our results do also confirm that there is evidence of a relationship between increased *EGFR* GCN and cetuximab efficacy.

Although *EGFR* amplification is readily identifiable by FISH analysis, in keeping with previous series (6, 20, 21), this genomic aberration was detected only in 2 (2.2%) patients, both responding to cetuximab. This seems to reflect the current knowledge on the DiFi cell line, which is unique among several colorectal cancer cell lines because it displays both high-level *EGFR* amplification and high levels of EGFR protein expression (22). Importantly, the DiFi cell line, previously established from a patient with familial adenomatous polyposis (23), is highly sensitive to growth inhibition by the anti-EGFR mAbs C225 (24), cetuximab (4), and ICR62 (22). These data therefore favor the hypothesis of a mechanistic link between high-level *EGFR* amplification, strong protein expression, and mAb activity. Similar to Moroni et al. (4), in one patient we detected a regional pattern of *EGFR* amplification and overlapping strong protein expression, which well underscores the relationship with protein overexpression but also questions whether a single tumor cross section analysis can be representative of the whole tumor sample.

Beside amplification, which is restricted to a very small subset of patients, other genomic aberrations may be involved in

sensitivity to cetuximab. In fact, two recent studies (5, 6) have suggested the association between *EGFR* genomic gains, mainly resulting from chromosome 7 polysomy, and outcome after anti-EGFR mAbs. Two distinct cutoff points representative of increased GCN have been indicated to predict objective response. We sought to determine the performance of each of the aforementioned cutoff points and verify their applicability in an independent set of patients. However, for different diagnostic indices examined, we found overall low performances (Table 2). We also found that neither cutoff point thus far published would provide an adequate outcome prediction in terms of survival (Table 3). Three main reasons can explain these low performances: First, it is widely accepted that a validation set generally provides less optimistic results than the training set where an individual cutoff point is generated. Second, in the absence of guidelines, differences related to the scoring methodology are possible, thereby affecting the reproducibility of the assay as well as its diagnostic performances. Lastly, with respect to the cutoff point by Sartore-Bianchi et al. (5), due to technical issues discussed by Cappuzzo et al. (6), it might be the case that this specific cutoff point, generated on 2 μm-thick sections, is not reproducible in studies like ours, which considered thicker sections. Eventually, a third cutoff point, consisting of 2.83 mean GCN, was indicated by ROC analysis on our data set as the best predictor of response. However, although three different cutoff points have been retrieved, the 95% CIs around sensitivity and specificity yielded by each cutoff point also include the diagnostic indices yielded by other cutoff points, thus indicating that results from these three studies are consistent.

According to our ROC curve analysis generated on objective response, we found a nonsignificantly improved progression-free survival (Fig. 2) and a significantly longer overall survival in patients harboring increased mean *EGFR* GCN (Fig. 3). Conflicting results have been reported on the potentially prognostic role of *EGFR* GCN in colorectal cancer. In the study by Sartore-Bianchi et al. (5), *EGFR* GCN by FISH did not affect the progression-free survival of 34 patients receiving only best supportive care. Conversely, in a similar cohort of patients treated by cetuximab, *EGFR* GCN by quantitative PCR showed a significant relationship with overall survival, although in that study no other relationship with objective response or progression-free survival was detected (13). Further investigations in the setting of chemotherapy-alone trials will be needed to clarify this issue.

As suggested by previous studies on DNA ploidy (9), *EGFR* GCN within individual colorectal cancer tumors might be represented in an extremely heterogeneous fashion. Our data, summarized by the intraclass correlation within a tumor sample and within a patient, along with the respective SD, support these earlier findings and led us to explore the possibility that a small subgroup of cells, which would not be picked up by a global measure of GCN, would drive the response to cetuximab. However, despite the attempts to test several summaries of the GCN distribution other than the mean, we could not identify one that might provide better performance indices than the mean GCN itself. Additionally, the relation between the mean of the mean *EGFR* GCN and the SD obtained in patients with multiple tumor samples (Fig. 1) suggests that the heterogeneity of GCN is a function of the level of GCN. This would imply that samples from tumors with

overall low mean GCN are more homogeneous, making this specific FISH pattern easier to identify. Therefore, considering that in our experience and that of others (5, 6) the FISH assay yields a negative predictive value equal to 85.2%, these results suggest that the detection of low *EGFR* GCN would be particularly helpful to detect those patients less likely to respond to cetuximab-based treatments, thereby lowering the risk to exclude potentially responsive patients.

From a technical standpoint, the heterogeneity of GCN distribution may have relevant consequences even on the methodology followed for scoring. For instance, whereas in our study we took into account all scores deriving from four to five tumor areas, in samples with GCN heterogeneity, Cappuzzo et al. (6) elected to retain only the area showing the worst score. Clearly, these differences represent a call to standardize the interpretation of the FISH assay. With 38% paired samples being deemed discrepant for the FISH status in our series, the heterogeneous distributions of *EGFR* GCN affect the capability to reproduce predefined cutoff point values in multiple samples from the same tumor (Fig. 4). Consistent with these findings, two previous studies comparing primaries and related metastases again suggested that in non–small-cell lung cancer, the nonhomogeneous FISH patterns of *EGFR* GCN might yield discrepancies in 27% and 32% paired samples (25, 26). Of note, according to our data, there is no evidence to claim that a systematic difference of mean *EGFR* GCN between primary tumors and metastatic lesions could underlie these discrepancies.

KRAS mutations have been identified as markers of resistance to cetuximab in several studies (10–12) and are rapidly finding their way to clinical practice. We aimed to study the combined effect of this marker and *EGFR* GCN on response and survival on cetuximab-based therapy. The optimal cutoff point to predict response in the 58 *KRAS*-wild-type patients was ≥ 2.76 mean *EGFR* GCN, as compared with ≥ 2.83 in the whole patient population. The c-index obtained in the *KRAS*-wild-type patients (0.69) was very similar to the c-index obtained in the whole population (0.71), suggesting a lot of variance of the *EGFR* GCN even in the *KRAS*-wild-type patients. In the two models considered for objective response and overall survival prediction, analysis of *EGFR* GCN significantly adds information to the knowledge of *KRAS* status. Furthermore, the significant interaction of overall survival with *EGFR* GCN in *KRAS*-wild-type and *KRAS*-mutant patients unexpectedly revealed a very dismal prognosis among *KRAS*-mutant patients harboring high *EGFR* GCN. To our knowledge, this study is the first to show that activating *KRAS* mutations overcome the potentially favorable role of increased *EGFR* GCN in colorectal cancer patients treated with cetuximab. In non–small-cell lung cancer, subgroup analyses based on *KRAS* mutation status showed that adding the anti-EGFR agent erlotinib to chemotherapy not only fails to provide more benefit over chemotherapy alone but may even determine a detrimental effect in patients with *KRAS* mutations (27). Massarelli et al. (28) also reported a poorer outcome after tyrosine kinase inhibitors in *KRAS*-mutant non–small-cell lung cancer patients harboring increased GCN. Despite reaching statistical significance, the negative interaction we observed between *EGFR* GCN and *KRAS* mutations in patients treated with cetuximab is difficult to explain and, given the relatively small number of patients, should be interpreted with caution. These findings are hypotheses-generating and deserve confirmation in future series.

Given the major role played by *HER2* as a dimerization partner of the *EGFR* within the *HER* family of receptors (29), we tested *HER2* GCN as a potential marker to guide patient selection. In keeping with previous reports (18, 20, 21), we found a low rate of *HER2* amplification (5.2%), albeit always correlated to a positive *HER2* protein expression. Interestingly, we detected an inverse relationship between *HER2* gene amplification and cetuximab efficacy. This is in line with preclinical investigations in pancreatic cell lines, indicating that coexpression of *EGFR* and *HER2* might be associated with resistance to cetuximab (30). In fact, although cetuximab is known to promote *EGFR* internalization (31), this process can be evaded by *HER2-EGFR* heterodimers (32). Given the "drugability" of *HER2*-associated tyrosine kinase, combinations including anti-HER2 approaches to overcome resistance to cetuximab would be of interest in *HER2*-driven tumors.

In conclusion, our results confirm an association between increased *EGFR* GCN and outcome in colorectal cancer patients treated with cetuximab. However, our prospective assessment of the performance of reported cutoff points and our rigorous assessment of GCN heterogeneity force us to conclude that clinical decision making based on any of the cutoff points thus far published is not warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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