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EGFR gene copy number assessment from areas with highest EGFR expression predicts response to anti-EGFR therapy in colorectal cancer

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BACKGROUND: Only 40–70% of metastatic colorectal cancers (mCRCs) with wild-type (WT) *KRAS* oncogene respond to anti-epidermal growth factor receptor (anti-EGFR) antibody treatment. *EGFR* amplification has been suggested as an additional marker to predict the response. However, improved methods for bringing the *EGFR* analysis into routine laboratory are needed.
METHODS: The material consisted of 80 patients with mCRC, 54 of them receiving anti-EGFR therapy. *EGFR* gene copy number (GCN) was analysed by automated silver *in situ* hybridisation (SISH). Immunohistochemical EGFR protein analysis was used to guide SISH assessment.
RESULTS: Clinical benefit was seen in 73% of high ($\geqslant 4.0$) *EGFR* GCN patients, in comparison with 59% of *KRAS* WT patients. Only 20% of low *EGFR* GCN patients responded to therapy. A high *EGFR* GCN number associated with longer progression-free survival ($P < 0.0001$) and overall survival ($P = 0.004$). Together with *KRAS* analysis, *EGFR* GCN identified the responsive patients to anti-EGFR therapy more accurately than either test alone. The clinical benefit rate of *KRAS* WT/high *EGFR* GCN tumours was 82%.
CONCLUSION: Our results show that automated *EGFR* SISH, in combination with *KRAS* mutation analysis, can be a useful and easily applicable technique in routine diagnostic practise for selecting patients for anti-EGFR therapy.
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The major prognostic determinant for patients with advanced colorectal cancer (CRC) with non-resectable metastases is the response to systemic therapy (Cunningham *et al*, 2010). For part of these patients, recent advances, including anti-epidermal growth factor receptor (EGFR) therapy have added clinical benefit and extended the median survival time (Cunningham *et al*, 2004; Douillard *et al*, 2010; Grothey, 2010; Peeters *et al*, 2010).

Tumours harbouring activating mutations of *KRAS*, a signalling molecule downstream of EGFR, do no benefit from the anti-EGFR monoclonal antibodies cetuximab and panitumumab (Linardou *et al*, 2008; Allegra *et al*, 2009). In *KRAS* wild-type (WT) patients, on the other hand, the addition of cetuximab to cytotoxic treatment in first line improves the response rates with 16–24% compared with cytotoxic therapy alone. However, about 40% of the previously untreated (Bokemeyer *et al*, 2009; Chang *et al*, 2009; Van Cutsem *et al*, 2009) and about 60–70% of the previously treated (Moroni *et al*, 2005; Lievre *et al*, 2006, 2008; Chang *et al*, 2009) *KRAS* WT patients do not respond to anti-EGFR treatment combined with chemotherapy. Consequently, there is a need for predictive markers among the *KRAS* WT patients. Changes in molecules downstream of EGFR, in particular *BRAF* gene mutations, *PIK3CA* mutations and loss of expression of the PTEN tumour-suppressor protein appear to associate with resistance to anti-EGFR treatment (Laurent-Puig *et al*, 2009; Siena *et al*, 2009). However, even the combination of these is likely to identify only a minority of non-responsive *KRAS* WT patients (Laurent-Puig *et al*, 2009).

Unlike the EGFR protein expression level assessed by immunohistochemistry (IHC) (Cunningham *et al*, 2004; Saltz *et al*, 2004; Chung *et al*, 2005), an increased *EGFR* gene copy number (GCN) has been associated with a favourable response to anti-EGFR therapy among *KRAS* WT patients (Moroni *et al*, 2005; Lievre *et al*, 2006; Sartore-Bianchi *et al*, 2007; Cappuzzo *et al*, 2008). Fluorescence *in situ* hybridisation (FISH) technique has been used in most previous studies (Moroni *et al*, 2005; Sartore-Bianchi *et al*, 2007; Cappuzzo *et al*, 2008; Personeni *et al*, 2008; Scartozzi *et al*, 2009). The FISH results are challenging to interpret and the lack of standardisation of analytical methods and scoring systems may partly explain why the *EGFR* GCN evaluation has not been incorporated into the clinical practice yet (Martin *et al*, 2009).

Silver *in situ* hybridisation (SISH) is a technique that can be applied to automated detection of *EGFR* GCN and chromosome 7 (Chr-7) number. SISH-based *EGFR* GCN can be easily performed, because it can be analysed by conventional bright field light microscopy. In addition, the chromogen of SISH is very stable unlike fluorochromes in FISH. The aim of this study hwas to

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evaluate the predictive value of *EGFR* GCN and Chr-7 number assessed by SISH from areas with highest IHC reactivity in patients with metastatic or locally advanced CRC treated with anti-EGFR monoclonal antibody therapy. The correlation between *EGFR* GCN and EGFR protein expression, as determined by IHC, was also evaluated, since previous reports have been conflicting (Shia *et al*, 2005; Spindler *et al*, 2006; Frattini *et al*, 2007; Hemmings *et al*, 2009).

PATIENTS AND METHODS

Patients

This retrospective study comprises a series of 80 metastatic or locally advanced CRC patients, 62 of whom were treated with anti-EGFR therapy at the Turku University Hospital. In all, 50% of the patients had metastatic disease at the time of diagnosis. The median age of the patients at diagnosis was 60 years (range, 34–73). Patient characteristics and treatments are presented in Table 1. Ten of the treated patients had a mutation in the *KRAS* gene, as the anti-EGFR therapy was administered before establishment of the predictive value of *KRAS* testing. The treatment response could be reliably evaluated for 54 out of 62 (87%) of treated patients. Of those, 25 *KRAS* WT patients received cetuximab or panitumumab either as single therapy or irinotecan combination therapy in a chemorefractory phase of the disease ($\geqslant$third line therapy). The response to anti-EGFR treatment was evaluated by computed tomography or magnetic resonance imaging according to the Response Evaluation Criteria in Solid Tumours (Eisenhauer *et al*, 2009). The study was conducted in accordance with the Declaration of Helsinki. The clinical data were retrieved and histological samples collected and analysed with the endorsement of the National Authority for Medico-Legal Affairs.

Procedures

Formalin-fixed, paraffin-embedded samples with at least 30% of CRC cells were selected and analysed for *KRAS* point mutations within codons 12 and 13 with the DxS K-RAS mutation kit (DxS Ltd, Manchester, UK).

In all, 3μm sections were stained with two monoclonal antibodies against EGFR (VentanaMedical Systems/Roche Diagnostics, Tucson, AZ, USA). EGFR (clone 3C6) mAb is directed against the extracellular domain of human EGFR, and EGFR (clone 5B7) mAb against the internal domain of human EGFR. All 80 tumour specimens were stained with the 5B7 anti-EGFR antibody and 74 tumour samples with the 3C6 anti-EGFR antibody. Stainings were performed with BenchMark XT (Ventana/Roche) using *ultra*VIEW Universal DAB Detection Kit (Ventana/Roche). EGFR IHC was scored independently by three observers (OC, JS, and ML) blinded of the clinical information. Three scoring parameters were recorded: the highest (covering at least 10% of the tumour area), the most common staining intensity, and the localisation of staining (membranous, cytoplasmic or both). Four categories of staining intensity were used: 0 (negative), + (weak), + + (moderate), and + + + (strong, similar to the intensity of the epidermal basal layer). In cases of discordance, a consensus score was used.

EGFR gene was detected from 5μm sections with *EGFR* DNA Probe (Ventana/Roche) and Chr-7 from parallel sections with Chr-7 oligonucleotide Probe (Ventana/Roche). SISH was performed with the BenchMark XT using *ultra*VIEW SISH Detection Kit (Ventana/Roche). From each tumour *EGFR* GCN (number of copies of gene per cell) and Chr-7 number (number of copies of chromosome per cell) were analysed by two observers (ML and JS) from the area of highest IHC reactivity. Forty tumour cells with the highest number of copies were analysed from the *EGFR* SISH slides. In addition to the average *EGFR* GCN and Chr-7 number, *EGFR*/Chr-7 copy number ratio was assessed.

Table 1 Baseline characteristics of patients who underwent SISH for *EGFR* and chromosome 7 and analysis of *KRAS* gene mutational status (a) and the subgroup of these patients that received anti-EGFR therapy with evaluable treatment response and sufficient follow up data (b)

	(a) Eligible patients for *KRAS* mutational status analysis, *EGFR* and chromosome 7 SISH analysis ($n=80$)	**(b) Patients treated with anti-EGFR therapy ($n=54$)**	
	***KRAS* WT and MT, $n=80$**	***KRAS* WT, $n=44$**	***KRAS* MT, $n=10$**
	***n* (%)**	***n* (%)**	***n* (%)**
Sex			
Female	34 (42)	18 (40.9)	6 (60)
Male	46 (58)	26 (59.1)	4 (40)
Site of primary tumour			
Colon	51 (63.8)	32 (72.7)	6 (60)
Rectum	28 (35)	12 (27.3)	4 (40)
Unknown	1 (1.2)		
Metastatic sites			
Single	28 (35)	19 (43.2)	2 (20)
Multiple	52 (65)	25 (56.8)	8 (80)
Tumour differentiation grade			
Grade 1	11 (13.8)	6 (13.6)	1 (10)
Grade 2	50 (62.5)	28 (63.7)	6 (60)
Grade 3	13 (16.2)	6 (13.6)	2 (20)
Unknown	6 (7.5)	4 (9.1)	1 (10)
Follow-up data of the patients			
Alive with disease	16 (20)	10 (22.7)	—
Alive and free of disease	5 (6.2)	1 (2.3)	—
Died of disease	59 (73.8)	33 (75)	10 (100)
KRAS mutational status			
KRAS WT	54 (67.5)	44 (100)	—
KRAS MT	24 (30)	—	10 (100)
Not evaluable	2 (2.5)	—	—
Anti-EGFR treatment			
Cetuximab	51 (63.8)	35 (79.5)	10 (100)
Panitumumab	10 (12.5)	8 (18.2)	—
Both	1 (1.2)	1 (2.3)	—
None	18 (22.5)	—	—
Line of therapy			
First	8 (12.9)	5 (11.4)	1 (10)
Second	14 (22.6)	12 (27.3)	—
Third or more	40 (64.5)	27 (61.3)	9 (90)
Anti-EGFR combination therapy			
Anti-EGFR combined to IRI	46 (74.2)	32 (72.7)	9 (90)
Anti-EGFR combined to OXA	10 (16.1)	8 (18.2)	1 (10)
Anti-EGFR combined to CAP	2 (3.2)	1 (2.3)	—
Single treatment	4 (6.5)	3 (6.8)	—

Abbreviations: CAP = capecitabine; EGFR = epidermal growth factor receptor; IRI = irinotecan; MT = mutated; OXA = oxaliplatin; SISH = silver *in situ* hybridization; WT = wild type.

FISH analysis with Vysis *EGFR*/CEP 7 FISH Probe Kit (Abbott Molecular Inc., Des Plaines, IL, USA) was performed on nine samples selected based on *EGFR* SISH results (three samples with clusters, three samples with more than four copies, and three samples with normal two copies), using standard protocols.

Statistical analysis

Statistical analyses were performed with the SAS 9.2 and Enterprise Guide 4.2 programs (SAS Institute Inc., Cary, NC, USA). Frequency table data were analysed with the χ^2-test or Fisher's exact test. Spearman correlation coefficients were calculated when correlations were analysed. The optimal cut-off values for *EGFR* GCN and Chr-7 number were defined with the receiver operating characteristic (ROC) analysis generated on response to treatment (clinical benefit *vs* progressive disease (PD)). Kaplan–Meier and log-rank tests as well as Cox proportional hazards regression model were used for univariate survival analysis. When analysing progression-free survival (PFS), the survival time was calculated from the onset of anti-EGFR treatment until disease progression. When evaluating the overall survival (OS), the survival time was calculated from the onset of anti-EGFR therapy until death. Multivariate survival analysis was carried out by using Cox's proportional hazards regression model. All statistical tests were two-sided. *P*-values <0.05 were considered to be statistically significant.

RESULTS

EGFR IHC and *EGFR* and Chr-7 SISH analysis

Owing to the chromogenic detection method of *EGFR* GCN and EGFR protein, it was possible to assess both parameters from identical tumour areas and to compare the results. The EGFR protein expression levels and subcellular localisations were examined by two different anti-EGFR antibodies: clone 5B7 against the intracellular domain and clone 3C6 against the extracellular domain, hereafter referred to as intracellular and extracellular domain antibodies, respectively. In general, the intensity and subcellular localisation of IHC reactivity showed considerable intratumoural variation with both antibodies (Figure 1). Therefore, the following parameters were determined: localisation, highest, and most common intensity. The results obtained with the two different antibodies statistically significantly correlated with each other disregarding the parameter used ($P<0.0001$, Spearman). The most intense areas were scored as moderate ($++$) in a majority of the tumours, while only one-tenth of the tumours showed areas of strong intensity ($+++$). The most common EGFR staining intensity was low ($+$) with both antibodies. The frequencies of these parameters are presented in Table 2.

The marked variation in EGFR expression as analysed by IHC might reflect an intratumoural variation in the *EGFR* GCN. Therefore, we assessed the *EGFR* GCN and Chr-7 number from areas with strongest EGFR staining. The mean *EGFR* GCN was 5.5 (median 5.5) and the mean Chr-7 number 5.4 (median 5.3).

The optimal cut-off values for *EGFR* GCN and Chr-7 number as determined with ROC curves were 4.0 (sensitivity 86%, specificity 72%, AUC 83%) and 4.5 (sensitivity 84%, specificity 79%, AUC 85%), respectively. The optimal cut-off value for *EGFR* GCN was in addition defined with ROC analysis for the selected patients with chemorefractory disease who received anti-EGFR therapy ± irinotecan in ⩾third line. The cut-off value proved to be 4.0 (sensitivity 89%, specificity 67%, AUC 84%) in this patient group as well. In all, 51 tumours out of 80 (64%) had an *EGFR* GCN above cut-off value determined by ROC-analysis (⩾4.0). The *EGFR* GCN analysis by SISH could not be performed in 2 out of 80 (2.5%) of the cases. Chr-7 number was above the cut-off value (⩾4.5) in 48 out of 80 (60%) of the tumours. The highest *EGFR*/Chr-7 GCN ratio was 2.8 (mean 1.05, median 1.0). The *EGFR* FISH results from nine selected tumours correlated with the SISH results.

An increased *EGFR* GCN and Chr-7 number correlated positively with EGFR IHC analysed by the intracellular domain antibody (Spearman, $P=0.01$ for both) (Table 3). The correlation remained statistically significant when the staining intensity (IHC) was dichotomised into categories 0 and $+$ *vs* $++$ and $+++$. A significant correlation between extracellular domain antibody reactivity and an increased Chr-7 number was seen (Spearman, $P=0.04$), whereas, no correlation was observed between extracellular domain antibody reactivity and *EGFR* GCN. The subcellular localisation of the EGFR IHC (intracellular and extracellular domain antibodies) did not correlate with *EGFR* GCN or the Chr-7 number. *KRAS* mutational status did not correlate either with *EGFR* and Chr-7 SISH or EGFR IHC results.

EGFR SISH and treatment response

In all, 73% of high *EGFR* GCN (⩾4.0) patients showed clinical benefit (complete response (CR) + partial response (PR) + stable disease (SD)) from anti-EGFR therapy, whereas only 20% of low *EGFR* GCN (<4.0) benefited from treatment (Figure 2). In comparison, 59% of the *KRAS* WT patients showed clinical benefit. In *KRAS* WT patients with a high *EGFR* GCN (⩾4.0), clinical benefit was more frequent (82%) than in the overall *KRAS* WT or high *EGFR* GCN population. A high Chr-7 number (⩾4.5) was also significantly associated with an improved anti-EGFR treatment response among *KRAS* WT patients.

Anti-EGFR drugs were given as first-line treatment to five *KRAS* WT patients, four of which (80%) showed an objective response. Interestingly, all four patients had an *EGFR* GCN ⩾4.0. The fifth *KRAS* WT patient had an *EGFR* GCN <4.0 and progressed during therapy. We performed the statistical analyses separately by excluding the five *KRAS* WT patients who received anti-EGFR therapy as first-line treatment. Improved response rates were still seen in the group of *KRAS* WT patients with a high *EGFR* GCN (⩾4.0); an objective response was observed in 25% (6 out of 24), SD in 54% (13 out of 24) and PD in 21% (5 out of 24) of the patients. In the patients with a low *EGFR* GCN (<4.0), progressive disease was seen in 80% (12 out of 15) of the cases (Fisher's exact test, $P=0.002$).

In addition, the statistical analyses were performed separately for the *KRAS* WT chemorefractory CRC patients who received anti-EGFR therapy in ⩾third line, either as single drug therapy ($n=3$) or in combination with irinotecan ($n=22$). In all, 84% of the patients with a high *EGFR* GCN (⩾4.0) achieved either a SD or PR. In contrast, the clinical benefit rate was only 33% for the patients with a low *EGFR* GCN (<4.0) (Fisher's exact test, $P=0.03$). Stable disease was the best response recorded for 13 out of 25 patients in this selected patient group and of those 69% (9 out of 13) had a prolonged SD (⩾24 weeks). When excluding the patients with SD duration of <24 weeks from the analysis a significant association between treatment response and *EGFR* GCN status was still seen in a similar fashion (Fisher's exact test, $P=0.02$).

EGFR SISH and survival

In the entire treated population, the *EGFR* GCN associated significantly with an improved PFS when using the ROC-curve based cut-off value of 4.0. Interestingly, the PFS time of the *KRAS* WT patients with *EGFR* GCN <4.0 was indifferent from those with *KRAS* mutation. The median PFS time of *KRAS* WT/*EGFR* GCN ⩾4.0 was 35 weeks compared with only 12 weeks of the *KRAS* WT/*EGFR* GCN <4.0 patients. The PFS remained significantly longer in the *KRAS* WT patient population with a high *EGFR* GCN when analysing only the patients treated with anti-EGFR therapy in second line or more (log-rank test, $P<0.0001$). Furthermore, in the

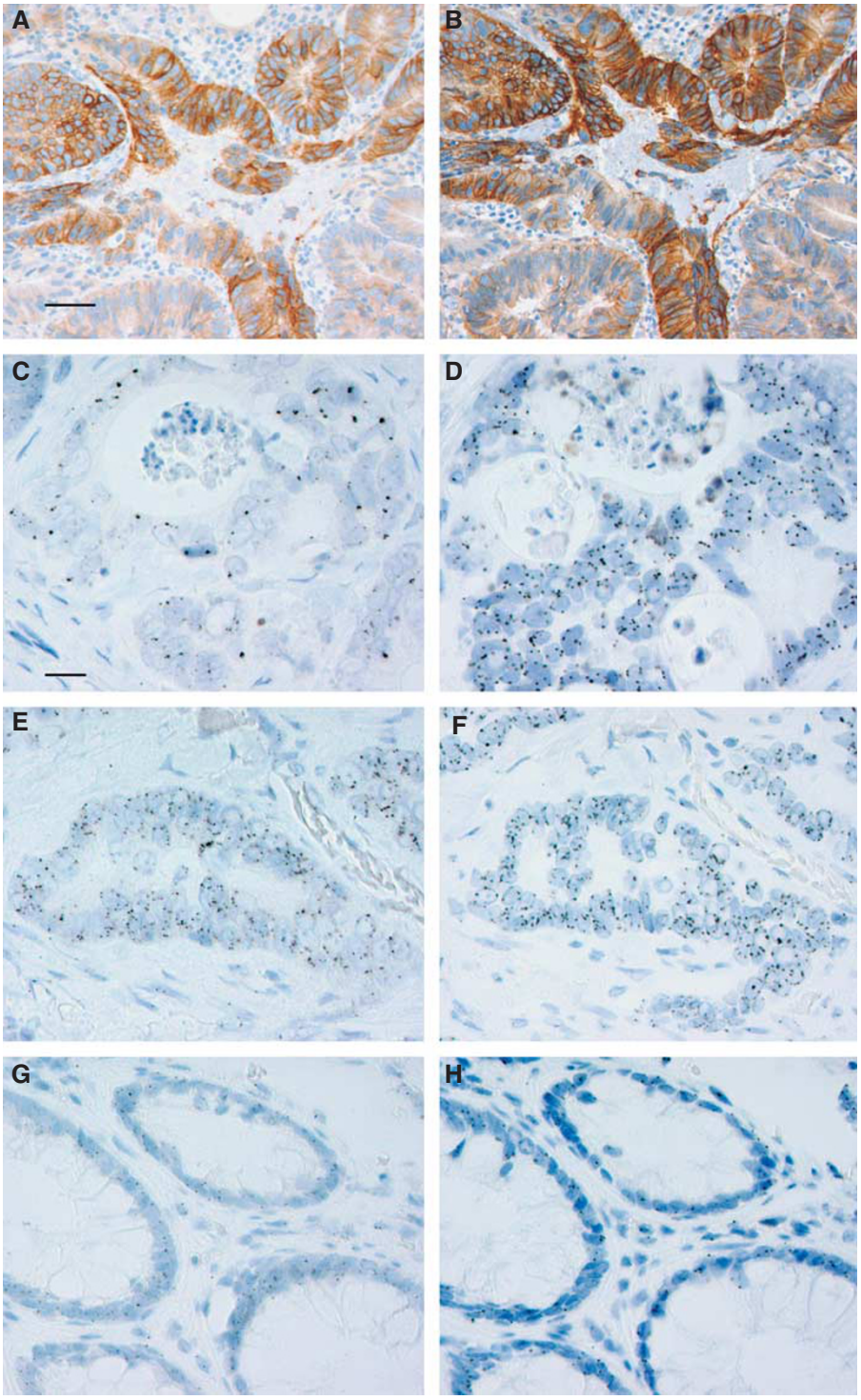

Figure 1 Epidermal growth factor receptor immunohistochemistry, *EGFR*, and Chr-7 SISH in colorectal cancer and normal colorectal tissues. Epidermal growth factor receptor IHC with clones 5B7 (**A**) and 3C6 (**B**). *EGFR* SISH revealing gene clusters (**C**) and the corresponding Chr-7 SISH (**D**). *EGFR* SISH with GCN $\geqslant$4.0 (**E**) and the corresponding Chr-7 SISH (**F**). *EGFR* SISH (**G**) and Chr-7 SISH (**H**) in normal colorectal tissue. Scale bar 0.05 mm (**A**, **B**), 0.02 mm (**C**–**H**).

cohort of chemorefractory patients treated either with single panitumumab or cetuximab ± irinotecan in $\geqslant$ third line ($n=25$), the median PFS time was significantly longer in the *KRAS* WT/*EGFR* GCN $\geqslant$4.0 patients than in the *KRAS* WT/*EGFR* GCN $<$4.0 patients; 35 *vs* 10 weeks (log-rank test, $P=0.003$; Cox test, $P=0.007$, HR: 0.22, 95% CI: 0.08–0.66). Similar results were obtained when excluding the patients with a short SD duration ($<$24 weeks) from the analysis (log-rank test, $P=0.0008$; Cox test, $P=0.003$, HR: 0.15, 95% CI: 0.04–0.53; PFS time 42 *vs* 8 weeks). Other factors associated with improved PFS in the entire group of

Table 2 EGFR protein expression assessed by anti-EGFR clone 5B7 ($n=80$) and anti-EGFR clone 3C6 antibodies ($n=74$)

	5B7 (H)	5B7 (C)	3C6 (H)	3C6 (C)
Intensity				
Negative	0 (0)	11 (13.8)	9 (12.2)	31 (41.9)
1+	19 (23.8)	50 (62.5)	20 (27.0)	37 (50.0)
2+	53 (66.2)	19 (23.7)	38 (51.3)	6 (8.1)
3+	8 (10)	0 (0)	7 (9.5)	0 (0)
Localisation				
Membranous	23 (28.75)	11 (13.75)	24 (32.4)	11 (14.9)
Cytoplasmic	23 (28.75)	46 (57.5)	18 (24.3)	28 (37.8)
Both	34 (42.5)	12 (15)	23 (31.1)	4 (5.4)
Negative	0 (0)	11 (13.75)	9 (12.2)	31 (41.9)

Abbreviations: C = most common staining; EGFR = epidermal growth factor receptor; H = highest staining. Values are given *n* (%).

Table 3 Correlations of *EGFR* GCN (SISH), Chr-7 number (SISH), *KRAS* status and EGFR protein expression (IHC), $n=74$ (*P*-values, Spearman)

	KRAS status	*EGFR* GCN (SISH) continuous variable	Chr-7 (SISH) continuous variable
Anti-EGFR clone 5B7, intensity			
Highest†	NS	0.01*	0.01*
Most common†	NS	NS	NS
Positive or negative≠	NS	0.01*	0.04*
Anti-EGFR clone 3C6, intensity			
Highest†	NS	NS	0.04*
Most common†	NS	NS	NS
Positive or negative≠	NS	NS	NS
Localisation			
5B7§	NS	NS	NS
3C6§	NS	NS	NS
EGFR *GCN (SISH)*			
Continuous variable	NS	—	<0.0001*
Cut-off 4.0	NS	—	—
Chr-7 number (SISH)			
Continuous variable	NS	<0.0001*	—
Cut-off 4.5	NS	—	—

Abbreviations: Chr-7 = chromosome-7; EGFR = epidermal growth factor receptor; GCN = gene copy number; IHC = immunohistochemistry; NS = not significant; SISH = silver *in situ* hybridisation; *Significant *P*-value; †0, 1+, 2+, or 3+; ≠Positive 2+ or 3+, negative 0, or 1+; §Membranous, cytoplasmic, both cytoplasmic and membranous or negative.

anti-EGFR treated patients were tumour differentiation grade (log-rank test, $P=0.001$) and the absence of *KRAS* gene mutation (log-rank test, $P=0.01$).

The *EGFR* GCN ⩾4.0 associated significantly with improved OS (log-rank test, $P=0.004$) in the entire treated population and in the subgroup of *KRAS* WT patients (log-rank test, $P=0.001$). The Chr-7 number did not associate with OS. The median OS time for patients with *KRAS* WT/*EGFR* GCN ⩾4.0 tumours was 85 weeks compared with 19 weeks for those with *KRAS* WT/*EGFR* GCN below the cut-off value. When excluding the patients treated with anti-EGFR therapy in first line the OS was still significantly higher in the patients with an *EGFR* GCN ⩾4.0 (log-rank test, $P=0.001$). In the selected patient group treated with anti-EGFR antibodies ± irinotecan in ⩾third line an *EGFR* GCN ⩾4.0 predicted a

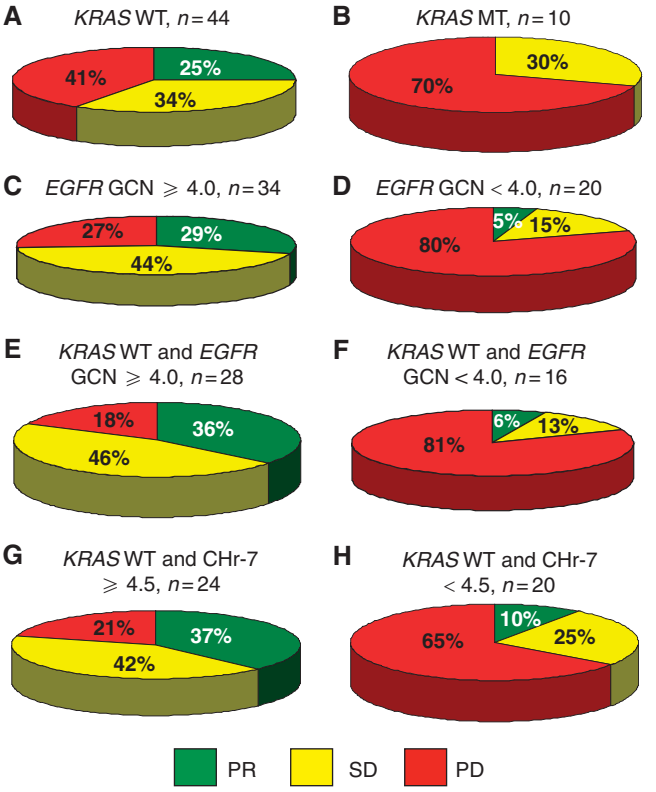

Figure 2 Response to anti-EGFR therapy according to *EGFR* GCN, Chr-7 number, and *KRAS* status (**A**–**H**).

prolonged OS as well; 74 *vs* 16 weeks in the group of patients with a low *EGFR* GCN (log-rank test, $P=0.0005$; Cox test, $P=0.003$, HR: 0.13, 95% CI: 0.03–0.49). The results remained significant when the patients with a short SD duration (<24 weeks) were excluded from the analysis (log-rank test, $P=0.0003$; Cox test, $P=0.004$, HR: 0.08, 95% CI: 0.01–0.44; PFS time 89 *vs* 14 weeks).

The responses, survival times and *P*-values are summarised in Table 4, survival curves shown in Figure 3.

Multivariate survival analysis

Variables that in univariate survival analysis significantly associated with PFS and OS in the anti-EGFR treated patient group were included in the Cox's multivariate analysis. The multivariate analysis for PFS included *EGFR* GCN, tumour differentiation grade, and *KRAS* status. *EGFR* GCN ($P=0.0003$, HR: 0.22, 95% CI: 0.09–0.50), tumour differentiation grade ($P=0.02$, HR: 0.38, 95% CI: 0.16–0.88), and *KRAS* ($P=0.04$, HR: 0.44, 95% CI: 0.20–0.97) proved to be independent predictors of PFS. When the *KRAS* WT patients were analysed separately, only *EGFR* GCN ($P=0.0003$, HR: 0.16, 95% CI: 0.06–0.43) independently predicted PFS. *EGFR* GCN and tumour differentiation grade were entered for OS analysis. Both variables predicted OS: *EGFR* GCN ($P=0.02$, HR: 0.44, 95% CI: 0.22–0.86), tumour differentiation grade ($P=0.046$, HR: 0.43, 95% CI: 0.19–0.99). In the *KRAS* WT subgroup of patients, *EGFR* GCN remained as a statistically significant predictor of OS ($P=0.01$, HR: 0.35, 95% CI: 0.16–0.78).

DISCUSSION

This study shows that *EGFR* GCN analysis, when performed from areas with highest EGFR expression, is a highly promising method for predicting the efficacy of anti-EGFR therapy in locally

Table 4 Tumour response of patients with *KRAS* WT ($n = 54$) and KRAS MT ($n = 10$) metastatic or locally advanced colorectal cancer treated with anti-EGFR therapy according to ROC curve based cut-off values of *EGFR* GCN and chromosome 7 number evaluated by SISH

	Treatment response					PFS					OS				
	Total no. of patients	PR	SD	PD	*P*-value Fisher's exact test	PFS time median (days)	*P*-value log-rank test	*P*-value Cox test[a]	HR	95% CI	OS time median (days)	*P*-value log-rank test	*P*-value Cox test[a]	HR	95% CI
KRAS WT and MT patients	54														
KRAS status															
KRAS WT	44	11 (25)	15 (34.1)	18 (40.9)	NS	151	**0.01**	**0.01**	0.40	0.20–0.84	352	0.3	0.3	0.67	0.32–1.38
KRAS MT	10	0	3 (30)	7 (70)		81					249				
EGFR GCN status															
EGFR GCN ⩾4.0	34	10 (29.4)	15 (44.1)	9 (26.5)	**0.0006**	224	**<0.0001**	**<0.0001**	0.21	0.10–0.43	483	**0.004**	**0.006**	0.41	0.22–0.77
EGFR GCN <4.0	20	1 (5)	3 (15)	16 (80)		84					134				
KRAS WT patients															
EGFR GCN															
⩾4.0	28	10 (35.7)	13 (46.4)	5 (17.9)	**0.0002**	244	**<0.0001**	**<0.0001**	0.17	0.07–0.39	598	**0.001**	**0.002**	0.32	0.16–0.66
<4.0	16	1 (6.2)	2 (12.5)	13 (81.3)		84					134				
Chromosome 7 number															
⩾4.5	24	9 (37.5)	10 (41.7)	5 (20.8)	**0.009**	214	0.2	0.2	0.67	0.35–1.29	520	0.1	0.1	0.56	0.28–1.13
<4.5	20	2 (10)	5 (25)	13 (65)		94					225				
KRAS MT patients															
EGFR GCN															
⩾4.0	6	0	2 (33.3)	4 (66.7)	NS	94	NS				369	NS			
<4.0	4	0	1 (25)	3 (75)		77					134				
Chromosome 7 number															
⩾4.5	6	0	2 (33.3)	4 (66.7)	NS	94	NS				369	NS			
<4.5	4	0	1 (25)	3 (75)		77					134				

Abbreviations: CI = confidence interval; CR = complete response; EGFR = epidermal growth factor receptor; GCN = gene copy number; HR = hazards ratio; MT = mutated; NS = not significant; OS = overall survival; PD = progressive disease; PFS = progression-free survival; PR = partial response; ROC = receiver operating characteristic; SD = stable disease; SISH = silver *in situ* hybridisation; WT = wild type. [a]Cox proportional hazards regression model. Treatment response values are given *n* (%). Significant *P*-values are shown in bold type.

advanced or metastatic CRC. Together with *KRAS* analysis *EGFR* GCN identifies the responsive patients more accurately than either test alone. In all, 73% of patients with a high *EGFR* GCN (⩾4.0) responded to anti-EGFR therapy, whereas a clear majority (80%) of the patients with a low *EGFR* GCN were non-responders. In comparison, 41% of the *KRAS* WT patients did not respond to treatment.

Previous reports, in which chromogenic ISH (CISH) and FISH were used to evaluate the *EGFR* GCN and/or Chr-7, have provided evidence for the association of increased *EGFR* GCN and response to anti-EGFR treatment. However, the predictive value of our study seems to be better than those (Moroni *et al*, 2005; Lievre *et al*, 2006; Frattini *et al*, 2007; Sartore-Bianchi *et al*, 2007; Cappuzzo *et al*, 2008; Personeni *et al*, 2008). What could be the explanation for this difference? One potential factor may be the use of IHC to guide the selection of the area for *in situ* analysis. The EGFR expression showed marked intratumoural variation and therefore, IHC was used to indicate the strongest EGFR immunoreactivity for evaluation of the *EGFR* GCN and Chr-7 number by SISH. This protocol might also explain why the *EGFR* GCN values were higher in our study than in most other studies reported. Another possible explanation could be the usage of a different *EGFR* probe. However, as the FISH analyses of nine selected cases were in concordance with SISH results, this is an unlikely explanation.

Methodological difficulties as well as reproducibility concerns have until now prevented the usage of *EGFR* GCN as a predictive marker in the clinic. The fully automated SISH technique offers several advantages compared with manually performed FISH and CISH. Automation improves reproducibility and compared with FISH, SISH enables morphological identification of the analysed tissue, which facilitates the interpretation (Dietel *et al*, 2007).

Several studies have indicated that EGFR IHC does not predict the response to EGFR-targeted therapies (Cunningham *et al*, 2004; Saltz *et al*, 2004; Chung *et al*, 2005; Cappuzzo *et al*, 2008). In addition, the correlation between EGFR IHC and *EGFR* GCN has been poor (Shia *et al*, 2005; Spindler *et al*, 2006; Frattini *et al*, 2007). Here, EGFR IHC with intracellular domain 5B7 antibody showed a significant correlation with the *EGFR* GCN and Chr-7 number. Our results may be due to the properties of the antibodies used. The novel 5B7 antibody detects the functionally active intracellular domain of EGFR, whereas other commercially available antibodies bind to the external domain of the EGFR. However, also IHC scoring method may have a role, the highest intensity assessment providing the best correlation with *EGFR* GCN. Typically, a constant intensive membranous staining correlated with areas of *EGFR* amplification. Consequently, although IHC does not predict treatment response, it is important for guiding SISH analysis, that is, indicating tumour areas with highest degree of *EGFR* GCN.

Currently, patients with metastatic CRC are screened for *KRAS* status and only those with *KRAS* WT tumours receive anti-EGFR therapy. This selection is not absolute and about half of the patients with *KRAS* WT tumours will receive the anti-EGFR monoclonal antibodies unnecessarily. Although, *BRAF*, *PIK3CA*/PTEN, and *NRAS* alterations explain a fraction of unresponsiveness (Laurent-Puig *et al*, 2009; Bardelli and Siena, 2010; De Roock *et al*, 2010) the search for further predictive markers in this setting is feasible. Improved predictive testing would minimise the risk of exposing the patients to harmful side-effects caused by EGFR targeted therapies and at the same time reduce the healthcare costs.

Our results suggest that cetuximab and panitumumab should not be offered to *KRAS* WT patients with *EGFR* GCN <4.0. Furthermore, according to our results, the analysis of *EGFR* GCN by SISH could in certain cases be used as a substitute for *KRAS* analysis, for example, when only a small biopsy of the tumour has been taken and the amount of tumour tissue is insufficient for *KRAS* analysis.

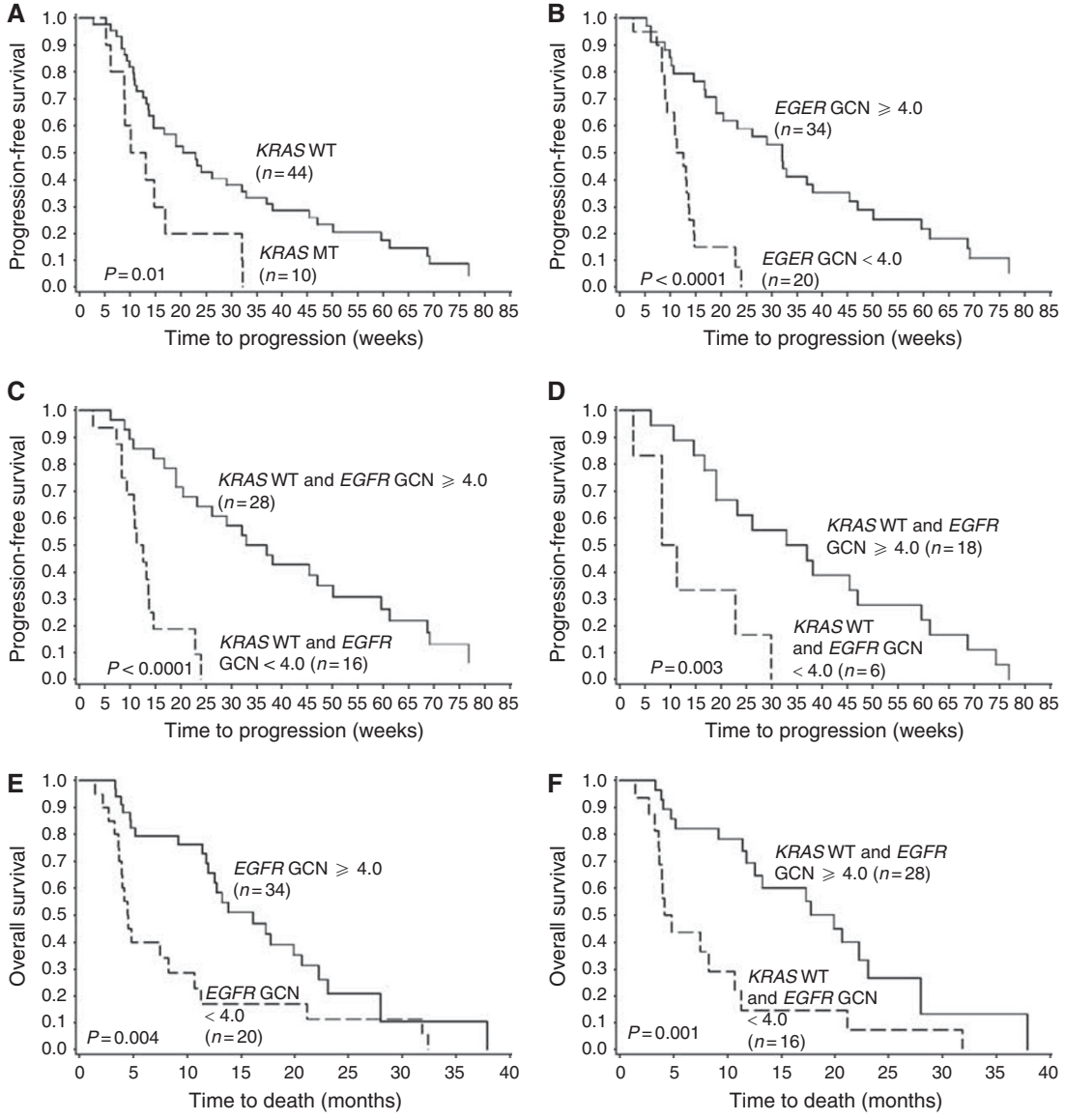

Figure 3 Kaplan–Meier curves for PFS (**A**–**D**) and OS (**E**–**F**). Progression-free survival in anti-EGFR treated patients by (**A**) *KRAS* and (**B**) *EGFR* gene copy number (GCN). (**C**) Progression-free survival in *KRAS* WT patients ($n=44$) according to *EGFR* GCN. (**D**) Progression-free survival according to *EGFR* GCN in selected chemorefractory *KRAS* WT patients treated with anti-EGFR therapy ± irinotecan in ⩾third line ($n=25$). (**E**) Overall survival by *EGFR* GCN. (**F**) Overall survival in *KRAS* WT patients according to *EGFR* GCN.

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