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. Gene copy number (GCN) was assessed 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 (≥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 practice 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 (Linaford 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 was to...
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 (≥ 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, 5 μm 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 ultraVIEW 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 5 μm 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 ultraVIEW 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)

|                      | KRAS WT and MT, n = 80 | KRAS WT, n = 44 | KRAS MT, n = 10 |
|----------------------|------------------------|----------------|-----------------|
| 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 EGFRC/E7 FISH Probe Kit (Abbott Molecular Inc., Des Plaines, IL, USA) was performed on nine samples selected based on EGFRC 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 χ²-test or Fisher’s exact test. Spearman correlation coefficients were calculated when correlations were analysed. The optimal cut-off values for EGFRC 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 EGFRC and Chr-7 SISH analysis
Owing to the chromogenic detection method of EGFRC GCN and EGFRC protein, it was possible to assess both parameters from identical tumour areas and to compare the results. The EGFRC protein expression levels and subcellular localisations were examined by two different anti-EGFR antibodies: clone SB7 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 EGFRC staining intensity was low (+) with both antibodies. The frequencies of these parameters are presented in Table 2.

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

The optimal cut-off values for EGFRC 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 EGFRC 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 EGFRC GCN above cut-off value determined by ROC-analysis (≥4.0). The EGFRC 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 EGFRC/Chr-7 GCN ratio was 2.8 (mean 1.05, median 1.0). The EGFRC FISH results from nine selected tumours correlated with the SISH results.

An increased EGFRC GCN and Chr-7 number correlated positively with EGFRC 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 EGFRC GCN. The subcellular localisation of the EGFRC IHC (intracellular and extracellular domain antibodies) did not correlate with EGFRC GCN or the Chr-7 number. KRAS mutational status did not correlate either with EGFRC and Chr-7 SISH or EGFRC IHC results.

EGFRC SISH and treatment response
In all, 73% of high EGFRC 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 EGFRC 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 EGFRC GCN (≥4.0), clinical benefit was more frequent (82%) than in the overall KRAS WT or high EGFRC 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 EGFRC GCN ≥4.0. The fifth KRAS WT patient had an EGFRC 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 EGFRC 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 EGFRC 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 EGFRC GCN (≥4.0) achieved either a SD or PR. In contrast, the clinical benefit rate was only 33% for the patients with a low EGFRC 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 EGFRC GCN status was still seen in a similar fashion (Fisher’s exact test, P = 0.02).

EGFRC SISH and survival
In the entirely treated population, the EGFRC 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 EGFRC GCN <4.0 was indifferent from those with KRAS mutation. The median PFS time of KRAS WT/EGFRC ≥4.0 was 35 weeks compared with only 12 weeks of the KRAS WT/EGFRC <4.0 patients. The PFS remained significantly longer in the KRAS WT patient population with a high EGFRC 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
cohort of chemorefractory patients treated either with single panitumumab or cetuximab ± irinotecan in ≥ third line (n = 25), the median PFS time was significantly longer in the KRAS WT/EGFR GCN ≥ 4.0 patients than in the KRAS WT/EGFR GCN < 4.0 patients; 35 vs 10 weeks (log-rank 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, \( P = 0.008 \); 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

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 ≥ 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).
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)   |
| Location |         |         |         |         |
| 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*                               | 0.004*                           |
| 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 |                                     |                                  |
| Cut-off 4.0     | NS          | —                                   | <0.0001*                         |
| Chr-7 number (SISH) | Continuous variable |                                     |                                  |
| 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.

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

**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
Molecular Diagnostics

EGFR protocol might also explain why the et al FISH, SISH enables morphological identification of the analysed CISH. Automation improves reproducibility and compared with several advantages compared with manually performed FISH and marker in the clinic. The fully automated SISH technique offers EGFR have until now prevented the usage of concordance with SISH results, this is an unlikely explanation.

IHC was used to indicate the strongest EGFR immunoreactivity for expression showed marked intratumoural variation and therefore, EGFR evidence for the association of increased EGFR GCN identifies the responsive patients more accurately than either (2), 255 – 262

| Treatment response | P-value Fisher’s exact test | P-value log-rank test | HR | 95% CI | P-value Cox test | P-value Fisher’s exact test | P-value log-rank test | HR | 95% CI |
|--------------------|-----------------------------|----------------------|----|-------|-----------------|-----------------------------|----------------------|----|-------|
| Total no. of patients | PR | SD | PD | PR | SD | PD | PR | SD | PD | PR | SD | PD | PR | SD | PD |
| 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) | NS | 81 | | | | | | | | | |
| 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 | | | | | | | | | | |
| KRAS WT patients | | | | | | | | | | | | | | | |
| EGF 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 |
| EGF GCN <4.0 | 16 | 1 (6.2) | 2 (12.5) | 13 (81.3) | 84 | | | | | | | | | | |
| 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 | | | | | | | | | | |
| KRAS MT patients | | | | | | | | | | | | | | | |
| EGF GCN >4.0 | 6 | 0 | 2 (33.3) | 4 (66.7) | NS | 94 | NS | NS | NS | 369 | NS | | | | |
| EGF GCN <4.0 | 4 | 0 | 1 (25) | 3 (75) | 77 | | | | | | | | | | |
| Chromosome 7 number | | | | | | | | | | | | | | | |
| >4.5 | 6 | 0 | 2 (33.3) | 4 (66.7) | NS | 94 | NS | NS | NS | 369 | NS | | | | |
| <4.5 | 4 | 0 | 1 (25) | 3 (75) | 77 | | | | | | | | | | |

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.

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. *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 strong 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 andChr-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 bond 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, (B) EGF gene copy number (GCN). (C) Progression-free survival in KRAS WT patients (n = 44) according to EGR GCN. (D) Progression-free survival according to EGR GCN in selected chemorefractory KRAS WT patients treated with anti-EGFR therapy ± irinotecan in ≥ third line (n = 25). (E) Overall survival by EGR GCN. (F) Overall survival in KRAS WT patients according to EGR GCN.

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