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HER2 gene copy number status may influence clinical efficacy to anti-EGFR monoclonal antibodies in metastatic colorectal cancer patients

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Background: In metastatic colorectal cancer (mCRC), *KRAS* is the only validated biomarker used to select patients for administration of epidermal growth factor receptor (EGFR)-targeted therapies. To identify additional predictive markers, we investigated the importance of HER2, the primary EGFR dimerisation partner, in this particular disease.

Methods: We evaluated the *HER2* gene status by fluorescence *in situ* hybridisation (FISH) in 170 *KRAS* wild-type mCRC patients treated with cetuximab or panitumumab.

Results: Depending on *HER2* gene copy number status, patients showed three distinct cytogenetic profiles: 4% of patients had *HER2* gene amplification (R:*HER2*/CEP17 $\geqslant$ 2) in all neoplastic cells (*HER2*-all-A), 61% of patients had *HER2* gain due to polysomy or to gene amplification in minor clones (*HER2*-FISH + *), and 35% of patients had no or slight *HER2* gain (*HER2*-FISH −). These subgroups were significantly correlated with different clinical behaviours, in terms of response rate (RR; $P = 0.0006$), progression-free survival (PFS; $P < 0.0001$) and overall survival (OS; $P < 0.0001$). Patients with *HER2*-all-A profile experienced the worst outcome, patients with *HER2*-FISH − profile showed an intermediate behaviour and patients with *HER2*-FISH + * profile were related to the highest survival probability (median PFS in months: 2.5 *vs* 3.9 *vs* 7.6, respectively; median OS in months: 4.2 *vs* 9.7 *vs* 13, respectively).

Conclusion: *HER2* gene copy number status may influence the clinical response to anti-EGFR-targeted therapy in mCRC patients.

In the era of targeted therapies, monoclonal antibodies (MoAbs) directed against the epidermal growth factor receptor (EGFR/ErbB-1) have expanded the treatment options for metastatic colorectal cancer (mCRC) patients (García-Foncillas and Díaz-Rubio, 2010). Both cetuximab and panitumumab are active as single agents in chemorefractory metastatic disease as well as in combination with various chemotherapy regimens, but efficacy is restricted to patients with wild-type (wt) *KRAS* status (Chu, 2012).

The HER (ErbB) family consists of EGFR, HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4) and is responsible for cell proliferation and survival via the activation of the RAS/RAF/ERK and PI3K/PTEN/AKT pathways (Wells, 1999). Several studies have

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demonstrated that an increased *EGFR* gene copy number is related to the response to anti-EGFR agents, whereas the deregulation of downstream targets of the EGFR pathway (i.e., mutations in the *KRAS*, *NRAS*, *BRAF* or *PIK3CA* genes or loss of PTEN protein expression) accounts for the resistance to anti-EGFR MoAbs (Moroni *et al*, 2005; Frattini *et al*, 2007; Sartore-Bianchi *et al*, 2007; Cappuzzo *et al*, 2008; Scartozzi *et al*, 2009; De Roock *et al*, 2010; Van Cutsem *et al*, 2011). Despite this evidence, only *KRAS* testing is performed clinically to drive decisions about the use of anti-EGFR-targeted agents (www.ema.europa.eu; www.fda.gov). The presence of mutations in the *KRAS* gene designates the 30–40% of mCRC patients who are resistant to MoAbs. The characterisation of alterations occurring in additional candidate genes (*NRAS*, *BRAF*, *PIK3CA*, PTEN) may increase the negative predictive value up to 70%, but it is not sufficient to identify all resistant cases (De Roock *et al*, 2010; Blanke *et al*, 2011; Van Cutsem *et al*, 2011; www.ema.europa.eu; www.fda.gov).

In addition to homodimerization, EGFR can heterodimerize with the other members of the HER family, which, if altered, may influence the response to anti-EGFR agents. A few works have investigated the role of HER2, the primary EGFR dimerisation partner, in this process. In mCRC, two recent studies demonstrated that *HER2* gene amplification allows for the activation of downstream signalling even when cetuximab is bound to EGFR, thus leading to drug resistance (Bertotti *et al*, 2011; Yonesaka *et al*, 2011). Additionally, in lung cancer, increased *HER2* gene copy number may affect the sensitivity to the EGFR inhibitors gefitinib or erlotinib (Cappuzzo *et al*, 2005; Cappuzzo *et al*, 2007; Daniele *et al*, 2007; Hirsch *et al*, 2009).

On the basis of these data, we investigated whether *HER2* gene copy number status may influence the response to cetuximab or panitumumab therapy in a large cohort of mCRC patients.

PATIENTS AND METHODS

Study population. In an international consortium effort, we retrospectively analysed archival material and clinical data from a series of 396 adenocarcinomas from mCRC patients treated with cetuximab or panitumumab between 2004 and 2010. Cetuximab or panitumumab were administered as single agents or in combination with chemotherapy (in the last case in irinotecan-resistant patients).

Forty-eight cases were recruited at the Institute of Pathology of Locarno (Switzerland), 53 at the Civic Hospital of Livorno (Italy), 101 at the University Hospital Gasthuisberg of Leuven (Belgium) and 194 at the Hellenic Cooperative Oncology Group (HeCOG) and the Aristotle University School of Medicine of Thessaloniki (Greece). Some of the data on partial cohorts have been previously published for other purposes (Frattini *et al*, 2007; Personeni *et al*, 2008; De Roock *et al*, 2010). All patients were characterised for *KRAS* status. The analysis of the *KRAS* sequence (codon 12, 13 and 61 in exons 2-3) was performed locally according to the standard protocols for DNA extraction, amplification and sequencing (Frattini *et al*, 2007; De Roock *et al*, 2010).

Only patients with wt *KRAS* status and *HER2* gene status evaluation were selected for this study. The investigated cohort was made up of 170 patients. Response rate (RR), progression-free survival (PFS) and overall survival (OS) were available for 158 patients, 162 patients and 153 patients, respectively.

This study was undertaken after approval by the Internal Ethics Review Boards.

Clinical evaluation and tumour response criteria. WHO criteria (only in HeCOG series) or Response Evaluation Criteria In Solid Tumours (RECIST) were used to assess the tumour response. Responders were considered to be those patients who achieved a complete or partial response; non-responders were those with stable or progressive disease. PFS was calculated from the start of cetuximab or panitumumab administration until progressive disease or death, whereas OS was defined as the time from the start of cetuximab or panitumumab treatment until the last follow-up or death.

***HER2* FISH analysis.** *HER2* testing was performed by fluorescent *in situ* hybridisation (FISH) at the Institute of Pathology of Locarno (Switzerland) using the LSI *HER2-neu*/CEP17 probe (PathVysion, Abbott, Baar, Switzerland) on 3–4 μm thick formalin-fixed paraffin-embedded tissue sections, as previously described and according to the manufacturer's instructions (Frattini *et al*, 2007; Martin *et al*, 2012). FISH handling and interpretation were performed following the European Cytogeneticists Association (ECA) recommendations for FISH on histological sections of solid tumours (http://www.e-c-a.eu/). The signals' evaluation was performed independently by two trained readers (VM and FM) with superimposable results in nearly all the cases (98% concordance). When non-concordance occurred, additional cells were scored until an agreement was reached. A minimum of 100 morphologically clear, non-overlapping nuclei from at least 8–10 different areas were scored. Only experiments with at least 90% hybridisation efficiency were considered.

HER2 gene amplification was defined as the presence of a ratio (R) $\geqslant 2$ between the *HER2* and the CEP17 signals, according to the currently accepted criteria (Sauter *et al*, 2009). Cases were classified as *HER2* amplified (*HER2*-A) if the percentage of cells with *HER2* gene amplification was $\geqslant 10\%$ (Figure 1A) (Cappuzzo *et al*, 2005). Among *HER2*-A patients, those with *HER2* gene amplification in the entire tissue section ($\geqslant 90\%$ of the cells) were identified and placed in the 'all-A' group (*HER2*-all-A) (Figure 1B). *HER2* gene copy number gain (*HER2*-CNG) by polysomy was defined as the presence of $\geqslant 4$ copies of the *HER2* gene in $\geqslant 40\%$ of the cells (Figure 1C) (Cappuzzo *et al*, 2005). Patients with *HER2*-CNG and patients with *HER2*-A were grouped as *HER2*-FISH positive (*HER2*-FISH+). Those with no or low *HER2* gain ($\geqslant 4$ copies of *HER2* gene in $<40\%$ of the cells) and without *HER2*-A were designated as FISH negative (*HER2*-FISH−) (Figure 1D) (Cappuzzo *et al*, 2005).

***EGFR* FISH analysis.** In a subgroup of 39 patients, for which tissue sections were available, *EGFR* gene status was assessed by FISH with the LSI *EGFR*/CEP7 probe according to the manufacturer's instructions (Abbott). *EGFR* gene amplification was defined as the presence of a R$\geqslant 2$ between the *EGFR* signals and the CEP7 signals. Cases were considerd as amplified if $\geqslant 10\%$ of the cells showed *EGFR* gene amplification (*EGFR*-A). Patients were classified according to the *EGFR* gene copy number as *EGFR*-FISH+ (i.e., $\geqslant 4$ copies of the *EGFR* gene in $\geqslant 40\%$ of the cells or *EGFR* gene amplification in $\geqslant 10\%$ of the cells) or as *EGFR*-FISH− (i.e., $\geqslant 4$ copies of the *EGFR* gene in $<40\%$ of the cells) (Martin *et al*, 2009; Varella-Garcia *et al*, 2009; Sartore-Bianchi *et al*, 2012).

Statistical analyses. The primary endpoint of the study was to evaluate the impact of the *HER2* gene copy number status on the response to anti-EGFR MoAbs in terms of RR, PFS and OS. Chi-square test was conducted to assess the association between *HER2* gene status and the response to anti-EGFR MoAbs. The Fisher's exact test was used when expected counts were <5. The PFS and OS analyses were performed by *HER2* gene status according to the Kaplan–Meier method, and survival curves were compared using the log-rank test. In order to assess the magnitude of the effect of *HER2* gene status profile on PFS and OS, we performed the analysis of the Cox proportional-hazards regression model to estimate the hazard ratio (HR) with the corresponding 95% confidence intervals (95% CIs). The level of significance was set at $P<0.05$. The data were analysed using the SAS System V9.1 (SAS Institute Inc, Cary, NC, USA).

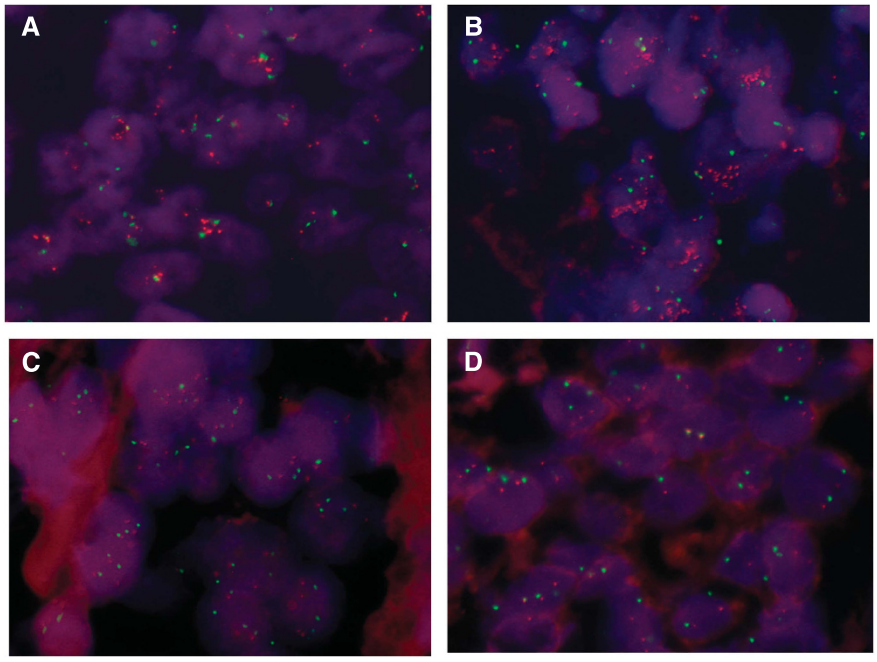

Figure 1. *HER2*/CEP17 FISH assay (red signal: *HER2* gene; green signal (CEP17): centromere of chromosome 17). (**A**) Tumour showing *HER2* gene amplification in a small population (30%) of the cells (classified as *HER2*-A case, minor-A profile); (**B**) tumour showing *HER2* gene amplification in all the cells (classified as *HER2-A* case, all-A profile); (**C**) tumor showing ⩾4 copies of *HER2* gene in >40% of the cells (CNG; classified as *HER2*-FISH +); and (**D**) tumour showing 2 balanced copies of *HER2* and CEP17 in >70% of the cells (classified as *HER2*-FISH −).

RESULTS

Patients. The majority of the patients were male (102 out of 170, 60%), and the median age was 62 years (range 28–76 years; Table 1). Cetuximab or panitumumab was given as a single agent in 12 patients (7%). In all the other cases ($N=158$, 93%), who experienced resistance to a previous irinotecan-based treatment, the anti-EGFR MoAb was given in combination with chemotherapy (Table 1). Twenty-three patients (13%) received anti-EGFR drugs as first-line treatment; 83 patients (49%) as second line, 49 patients (29%) as third line and 15 (9%) as fourth line or more.

In the entire cohort, the RR was 35% (complete response in 2% and partial response in 33% of the patients), the median PFS was 6.0 months and the median OS was 11.3 months.

***HER2* FISH analysis.** Out of the 170 eligible patients, 34 (20%) were classified as *HER2*-A (median R = 4.6) and 136 (80%) as non-amplified (*HER2*-non-A; Figure 2). Among the *HER2*-A patients, 7 patients (4% of the entire cohort) had amplification in ⩾90% of the tumour cells (*HER2*-all-A). The remaining *HER2*-A patients ($N=27$) showed amplification in minor clones, with a frequency of cells ranging from 10–60% (*HER2*-minor-A) (Figure 2). In the *HER2*-non-A group, 77 patients showed *HER2*-CNG due to polysomy (77 out of 170, 45%). Thus, 111 patients (65%) were classified as *HER2*-FISH +, accounting *HER2*-A and *HER2*-CNG (Figure 2). The remaining 59 (35%) patients of the *HER2*-non-A group showed no polysomy and no gene amplification and were classified as *HER2*-FISH − (Figure 2).

By separating cases with the *HER2*-all-A profile from the group of *HER2*-FISH + cases, 104 patients (61%) exhibited an increased number of *HER2* signals due to either *HER2* gene amplification in minor clones or to *HER2*-CNG. This last group was labelled '*HER2*-FISH +*' (Figure 2).

Correlation between HER2 gene copy number and response to anti-EGFR MoAbs. All statistical correlations are detailed in Tables 2 and 3.

Table 1. Patients' characteristics, treatment and response to anti-EGFR agents

Patients ($N=170$)		
Age (years)		
Mean ± s.d.	60 ± 11	
Median	62	
Range	28–76	
	Number of cases	**Percentage (%)**
Sex		
Male	102	60
Female	68	40
Regimens		
Cetuximab or panitumumab	12	7
Cetuximab + chemotherapy	158	93
Response ($N=158$)		
Complete response	3	2
Partial response	53	33
Non-responders	102	65

Abbreviation: EGFR, epidermal growth factor receptor.

The analyses of patients' outcome according to *HER2* gene status revealed that patients with *HER2*-all-A had significantly worse PFS ($P=0.0012$, Figure 3A) and OS ($P<0.0001$, Figure 3B) than those of the other patients (*HER2*-non-A and *HER2*-minor-A patients). In fact, the median PFS was 2.5 months for *HER2*-all-A patients and 6.7 months for the other patients, with an HR of 3.65 (95% CI: 1.57–8.46, $P=0.0026$; Table 2). The median OS was 4.2 months for *HER2*-all-A patients and 13 months for the other

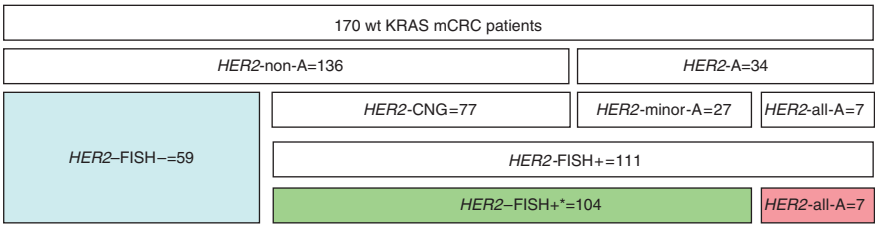

Figure 2. Patients' distribution depending on *HER2* gene status as detected by FISH. The detailed analysis of *HER2* gene copy number allows to recognise different groups of tumours with distinct cytogenetic features. *HER2*-A = *HER2* gene amplification (R:*HER2*/CEP17 ⩾ 2 in ⩾10% of the cells); *HER2*-all-A = *HER2* gene amplification in all the sample (⩾90% of cells); *HER2*-CNG = *HER2* gene copy number gain (presence of ⩾4 copies of the *HER2* gene in ⩾40% of the cells); *HER2*-FISH + = *HER2*-A and *HER2*-CNG; *HER2*-FISH + * = *HER2*-FISH + excluding *HER2*-all-A (i.e., *HER2*-CNG and *HER2*-minor-A); *HER2*-FISH − = absence of CNG and absence of *HER2* amplification; *HER2*-minor-A = *HER2* gene amplification in a minor population (10–60% of cells); *HER2*-non-A = absence of *HER2* gene amplification; mCRC = metastatic colorectal cancer; wt = wild type.

Table 2. Statistical analyses of correlation between *HER2* gene copy number status and efficacy in terms of progression free survival (PFS) and overall survival (OS)

	PFS			OS		
	N	**Median PFS (months)**	**HR (95% CI; *P*-value)**	***N***	**Median PFS (months)**	**HR (95% CI; *P*-value)**
All patients	162	6		153	11.3	
HER2-all-A *vs* others (HER2-non A and HER2-minor-A)	6 *vs* 156	2.5 *vs* 6.7	3.65 (1.57–8.46; ***P* = 0.0026**)	6 *vs* 147	4.2 *vs* 13	5.05 (2.17–11.77; ***P* = 0.0002**)
HER2-FISH + *vs* HER2-FISH −	107 *vs* 55	7.4 *vs* 3.9	2.00 (1.42–2.83; ***P* < 0.0001**)	100 *vs* 53	12.7 *vs* 9.7	1.18 (0.83–1.69; *P* = 0.3600)
HER2-all-A *vs* HER2-FISH − *vs* HER2-FISH + *	6 *vs* 55 *vs* 101	2.5 *vs* 3.9 *vs* 7.6		6 *vs* 53 *vs* 94	4.2 *vs* 9.7 *vs* 13	
HER2-all-A *vs* *HER2*-FISH −			2.28 (0.96–5.40; *P* = 0.0606)			4.36 (1.82–10.89; ***P* = 0.0010**)
HER2-all-A *vs* *HER2*-FISH + *			4.90 (2.08–11.55; ***P* = 0.0003**)			5.48 (2.33–12.92; ***P* < 0.0001**)
HER2-FISH − *vs* *HER2*-FISH + *			2.15 (1.51–3.05; ***P* < 0.0001**)			1.26 (0.87–1.81; *P* = 0.2167)

Abbreviations: CI = confidence interval; FISH = fluorescent *in situ* hybridization; *HER2*-all-A = *HER2* gene amplification in all the sample (R:*HER2*/CEP17 ⩾ 2 in ⩾90% of cells); *HER2*-FISH + *HER2* gene copy number gain (CNG: presence of ⩾4 copies of the *HER2* gene in ⩾40% of the cells) and *HER2*-A (HER2 gene amplification as R:*HER2*/CEP17 ⩾ 2 in ⩾10% of cells); *HER2*-FISH − = absence of CNG and absence of *HER2* amplification; *HER2*-minor-A = *HER2* gene amplification in a minor population (10–60% of cells); *HER2*-non-A: absence of *HER2* gene amplification; HR = hazard ratio. Significant *P*-values are in bold. HR with the corresponding 95% CI and *P*-values for PFS and OS were calculated using the analysis of the Cox proportional-hazards regression model.

patients, with an HR of 5.05 (95% CI: 2.17–11.77, $P = 0.0002$; Table 2). No difference in RR was observed between the two groups (33% *vs* 35%, $P = 1.0000$; Table 3).

On the contrary, patients with *HER2-FISH* + profile had a significant better RR ($P = 0.0004$, Table 3) and PFS ($P < 0.0001$, Figure 4) than with *HER2-FISH* − . The RR for *HER2*-FISH + patients was 45%, whereas for *HER2*-FISH − patients it was 17% (Table 3). The median PFS was 7.4 months for *HER2*-FISH + patients and 3.9 months for *HER2*-FISH − patients, with an HR of 2.00 (95% CI: 1.42–2.83, $P < 0.0001$; Table 2). No difference in OS was observed between these two groups (median OS: 12.7 months *vs* 9.7 months, HR: 1.18, 95% CI: 0.83–1.69, $P = 0.3600$; Table 2).

Interestingly, three different groups of patients with significant differences in RR ($P = 0.0006$, Table 3), PFS ($P < 0.0001$, Figure 5A) and OS ($P < 0.0001$, Figure 5B) were identified in relation to *HER2* gene copy number status, namely *HER2*-all-A, *HER2*-FISH − and *HER2*-FISH + * (Figure 5). The RRs for these three groups were 33, 17 and 46%, respectively ($P = 0.0006$, Table 3). *HER2*-all-A patients had the worst outcome, *HER2*-FISH − were in the middle and *HER2*-FISH + * showed the highest survival probability. The median PFS was 2.5 months for *HER2*-all-A patients, 3.9 months for *HER2*-FISH − and 7.6 months for *HER2*-FISH + * (Table 2). The median OS was 4.2 months for *HER2*-all-A patients, 9.7 months for *HER2*-FISH − and 13 months for *HER2*-FISH + * (Table 2).

***EGFR* FISH analysis and the correlation with *HER2* gene status.** Among the 39 patients analysed for *EGFR* gene status, 9 (23%) demonstrated *EGFR*-A. The *EGFR*-FISH + profile was observed in 30 patients (77%), whereas the *EGFR*-FISH − profile was detected in 9 patients (23%). Two patients (5%) had amplifications of both the *HER2* and *EGFR* genes (one patient was *HER2*-all-A and the other was *HER2*-minor-A). Although not statistically significant, the comparison of *HER2* gene status and *EGFR* gene status showed an interesting correlation ($P = 0.0648$, Table 4). The vast majority of the *HER2*-FISH + patients (18 out of 20, 90%) had a concomitant CNG of the *EGFR* gene (*HER2*-FISH + /*EGFR*-FISH +); the other two patients (10%) were *HER2* FISH + /*EGFR* FISH − . Among the 19 *HER2*-FISH − patients, 12 were *EGFR* FISH + (63%) and 7 were *EGFR*-FISH − (37%) (Table 4).

DISCUSSION

In mCRC, KRAS is the only validated biomarker used clinically to identify patients who are resistant to the anti-EGFR MoAbs cetuximab and panitumumab (Blanke *et al*, 2011). The alteration of other elements downstream of EGFR, such as the mutation of *BRAF*, *NRAS* and *PIK3CA* or the loss of PTEN expression, appears to mimic *KRAS* mutation, but the analysis of these markers is not being used at the clinical level because they have not been validated in independent studies yet (Frattini *et al*, 2007; Di Nicolantonio *et al*, 2008; Sartore-Bianchi *et al*, 2009; De Roock *et al*, 2010).

Scarce data are available regarding the deregulation of the EGFR family members HER2, HER3 and HER4, which dimerise with EGFR and thus potentially affect the efficacy of EGFR-targeted therapies. At the preclinical level, the amplification of the *HER2* gene has been shown to lead to cetuximab resistance in mCRC due to the continued activation of EGFR downstream pathways when cetuximab is bound to EGFR (Bertotti *et al*, 2011; Yonesaka *et al*, 2011).

We investigated the *HER2* gene status in a cohort of 170 *KRAS* wt mCRC patients treated with cetuximab or panitumumab. We

Table 3. Statistical analyses of correlation between *HER2* gene copy number status and clinical data in terms of response rate (RR)

RR			
All patients ***N*** **= 158**	**Responders** ***N*** **= 56**	**Non-responders** ***N*** **= 102**	***P*-value**
***HER2*-all-A *vs* others (*HER2*-non A and *HER2*-minor-A)**			
6	2 (33%)	4 (67%)	1.0000
152	54 (35%)	98 (65%)	
***HER2*-FISH + *vs* *HER2*-FISH −**			
104	47 (45%)	57 (55%)	**0.0004**
54	9 (17%)	45 (83%)	
HER2*-all-A *vs* *HER2*-FISH − *vs* *HER2*-FISH +			
6	2 (33%)	4 (67%)	
54	9 (17%)	45 (83%)	**0.0006**
98	45 (46%)	53 (54%)	

Abbreviations: FISH = fluorescent *in situ* hybridization; *HER2*-all-A = *HER2* gene amplification in all the sample (R:*HER2*/CEP17 ⩾2 in ⩾90% of cells); *HER2*-FISH + = *HER2* gene copy number gain (CNG: presence of ⩾4 copies of the *HER2* gene in ⩾40% of the cells) and *HER2*-A (HER2 gene amplification as R:*HER2*/CEP17 ⩾2 in ⩾10% of cells); *HER2*-FISH − = absence of CNG and absence of *HER2* amplification; *HER2*-minor-A = *HER2* gene amplification in a minor population (10–60% of cells); *HER2*-non-A = absence of *HER2* gene amplification. Significant *P*-values are in bold. *P*-values for RR were calculated using Chi-square test.

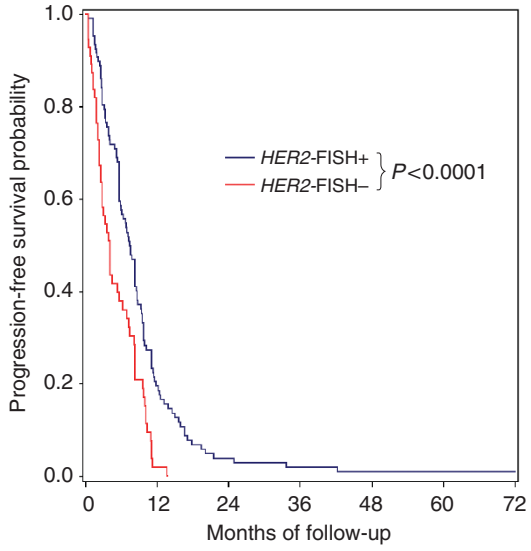

Figure 4. Kaplan–Meier curves for progression-free survival (PFS) of *HER2*-FISH + patients *vs* *HER2*-FISH − patients. PFS (median months): 7.4 (*HER2*-FISH +) *vs* 3.9 (*HER2*- FISH −), HR: 2.00, 95% CI: 1.42–2.83, $P<0.0001$. *HER2*-FISH + = *HER2* gene CNG (presence of ⩾4 copies of the *HER2* gene in ⩾40% of the cells) and *HER2*-A (*HER2* gene amplification as R:*HER2*/CEP17 ⩾2 in ⩾10% of cells); *HER2*-FISH − = absence of CNG and absence of *HER2* amplification; HR = hazard ratio; CI = confidence interval.

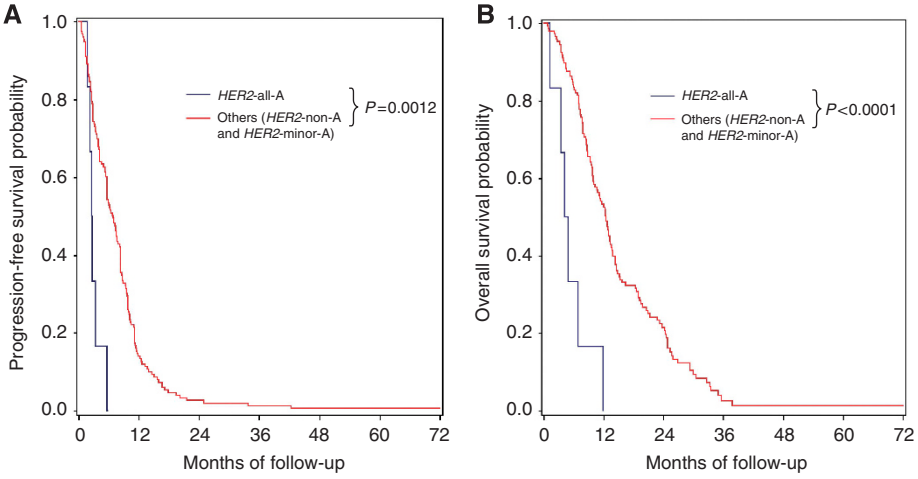

Figure 3. Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS) of *HER2*-all-A patients *vs* other patients (i.e., *HER2*-non-A and *HER2*-minor-A patients). (**A**) PFS (median months): 2.5 (*HER2*-all-A) *vs* 6.7 (*HER2* non A and *HER2*-minor-A), HR: 3.65, 95% CI: 1.57–8.46, $P=0.0026$. (**B**) OS (median months): 4.2 (*HER2*-all-A) *vs* 13 (*HER2* non A and *HER2*-minor-A), HR: 5.05, 95% CI: 2.17–11.77, $P=0.0002$. *HER2*-all-A = *HER2* gene amplification in all the sample (⩾90% of cells); *HER2*-minor-A = *HER2* gene amplification in a minor population (10–60% of cells); *HER2*-non-A = absence of *HER2* gene amplification; HR = hazard ratio; CI = confidence interval.

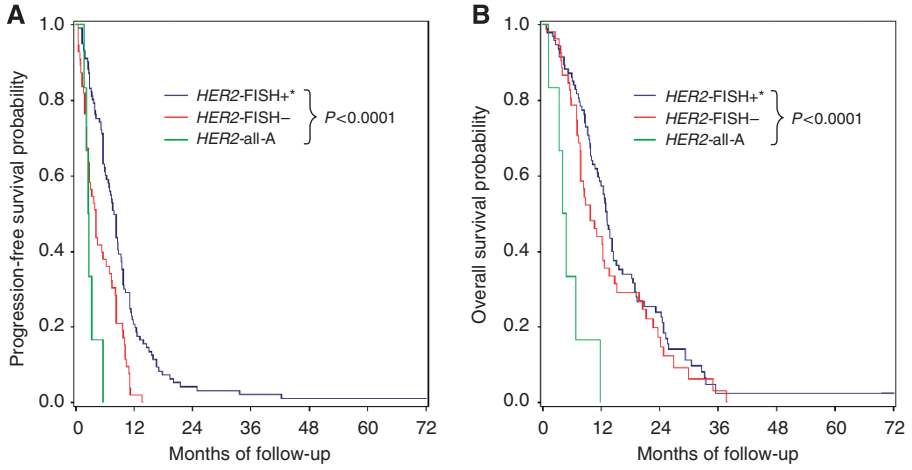

Figure 5. Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS) of *HER2*-all-A *vs* *HER2*-FISH + * *vs* *HER2*-FISH − patients. (**A**) PFS (median months): 2.5 (*HER2*-all-A) *vs* 3.9 (*HER2*-FISH −) *vs* 7.6 (*HER2*-FISH + *). *HER2*-all-A *vs* *HER2*-FISH − HR: 2.28, 95% CI: 0.96–5.40, $P = 0.0606$; *HER2*-all-A *vs* *HER2*-FISH + * HR: 4.9, 95% CI: 2.08–11.5, $P = 0.0003$; *HER2*-FISH − *vs* *HER2*-FISH + * HR: 2.15, 95% CI: 1.51–3.05, $P < 0.0001$. (**B**) OS (median months): 4.2 (*HER2*-all-A) *vs* 9.7 (*HER2*-FISH −) *vs* 13 (*HER2*-FISH + *). *HER2*-all-A *vs* *HER2*-FISH − HR: 4.36, 95% CI: 1.82–10.89, $P = 0.0010$; *HER2*-all-A *vs* *HER2*-FISH + * HR: 5.48, 95% CI: 2.33–12.92, $P < 0.0001$; *HER2*-FISH − *vs* *HER2*-FISH + * HR: 1.26, 95% CI: 0.87–1.81, $P = 0.2167$. *HER2*-all-A = *HER2* gene amplification in all the sample (R:*HER2*/CEP17 ⩾2 in ⩾90% of cells); *HER2*-FISH + * = *HER2*-FISH + excluding *HER2*-all-A; *HER2*-FISH − = absence of CNG and absence of *HER2* amplification; HR = hazard ratio.

detected *HER2* gene amplification in 20% of the patients (using R:*HER2*/CEP17 ⩾2 in ⩾10% of tumour cells as criteria, according to Cappuzzo *et al*, 2005). This frequency is higher if compared with previously published studies that demonstrated *HER2* gene amplification in 2–5% of mCRC cases (Al-Kuraya *et al*, 2007; Personeni *et al*, 2008; Bertotti *et al*, 2011; Yonesaka *et al*, 2011). The reasons for this difference may be related to the patients selection criteria (i.e., we considered only *KRAS* wt mCRC cases treated with MoAbs) and to discrepancy in evaluating FISH results. The interpretation of the *HER2* FISH signal might be challenging in mCRC due to its peculiar morphology (the cells are packed and overlapping) and to the amplification pattern, which has greater heterogeneity (Figure 1A) than the more familiar *HER2* gene amplification in breast cancer. In fact, if we consider only those cases with *HER2* gene amplification in almost the entire tissue section (⩾90% of the cells, *HER2*-all-A profile; Figure 1B), which are easier to recognise and are similar to the well known pattern frequently observed in breast cancer, we identified seven cases, corresponding to 4% of the entire cohort, which is consistent with the current literature.

By correlating the *HER2* gene status with data of response and follow-up, we observed the existence of three distinct subgroups of patients with peculiar behaviour and different bearing to anti-EGFR-MoAbs depending on *HER2* gene copy number as detected by FISH. These three groups can be easily recognised if FISH evaluation is performed by describing the pattern of signals (i.e., the abnormalities revealed) and their frequency (i.e., the percentage of the cells involved; Figure 6), an approach that is recommended in the investigation of cytogenetic markers by FISH in solid tumours (Tibiletti, 2007). These classes are represented by: patients whose tumours show *HER2* gene amplification in all the sample (*HER2*-all-A); patients with increased *HER2* gene copy number due to polysomy (*HER2*-CNG) or to *HER2* gene amplification in minor clones (*HER2*-minor-A); patients with no *HER2* imbalance (*HER2*-FISH −) (Figure 6).

Our results revealed that in *KRAS* wt mCRC patients, the *HER2*-all-A status conferred resistance to cetuximab or panitumumab, thereby confirming the preliminary findings of an independent study (Bertotti *et al*, 2011). In addition, the *HER2*-all-A profile affected PFS and OS (*HER2*-all-A patients had the worst PFS and OS), supporting recently published data (Yonesaka *et al*, 2011). By contrast, the *HER2*-minor-A and the *HER2*-CNG profiles (*HER2*-FISH + * status) might be more likely to respond to anti-EGFR therapies. In agreement with data from non-small-cell lung cancers treated with the EGFR tyrosine kinase inhibitors gefitinib or erlotinib (Cappuzzo *et al*, 2007; Daniele *et al*, 2007; Hirsch *et al*, 2009), these two cytogenetic pictures represent a good prognostic marker in terms of PFS and OS. On the contrary, the *HER2* normal status (FISH −) could stand for anti-EGFR therapies inefficacy (Figure 6).

Table 4. Patients' distributions and correlation depending on *HER2* gene status and *EGFR* gene status as detected by FISH

Gene status by FISH	***EGFR*-FISH +**	***EGFR*-FISH −**	**Total**
HER2-FISH +	18	2	20
HER2-FISH −	12	7	19
Total	30	9	39

Abbreviations: EGFR = epidermal growth factor receptor; FISH = fluorescent *in situ* hybridization *EGFR*-FISH + = *EGFR*-CNG (i.e., *EGFR* gene copy number gain defined as the presence of ⩾4 copies of the *EGFR* gene in ⩾40% of the cells) and *EGFR*-A (*EGFR* gene amplification); *EGFR*-FISH − = absence of CNG and absence of *EGFR* amplification; *HER2*-FISH + = *HER2*-CNG (i.e., *HER2* gene copy number gain defined as the presence of ⩾4 copies of the *HER2* gene in ⩾40% of the cells) and *HER2*-A (*HER2* gene amplification); *HER2*-FISH − = absence of CNG and absence of *HER2* amplification. $P = 0.0648$.

By investigating the three groups (*HER2*-all-A, *HER2*-FISH + *, *HER2*-FISH −) according to their lines of treatment, we did not observe any significant correlation, although the most of *HER2*-all-A patients were in the first or second line, whereas the most of *HER2*-FISH + * were in the second or third line (data not shown). Therefore, our data seem to indicate that the changes in terms of clinical efficacy among the groups depends on patients' genetic profile rather than to the length of treatment.

From the biological point of view, the difference in response to anti-EGFR therapy between *HER2*-all-A tumours and *HER2* FISH + * might stem from the differences in *HER2* gene deregulation. In cancers where the majority of cells carry gene amplification, the *HER2* oncogene is likely an essential driver of tumour growth. Therefore, *HER2*-all-A patients may be resistant because the majority of their cancer cells have the intrinsic ability to continuously activate downstream pathways (specifically upon

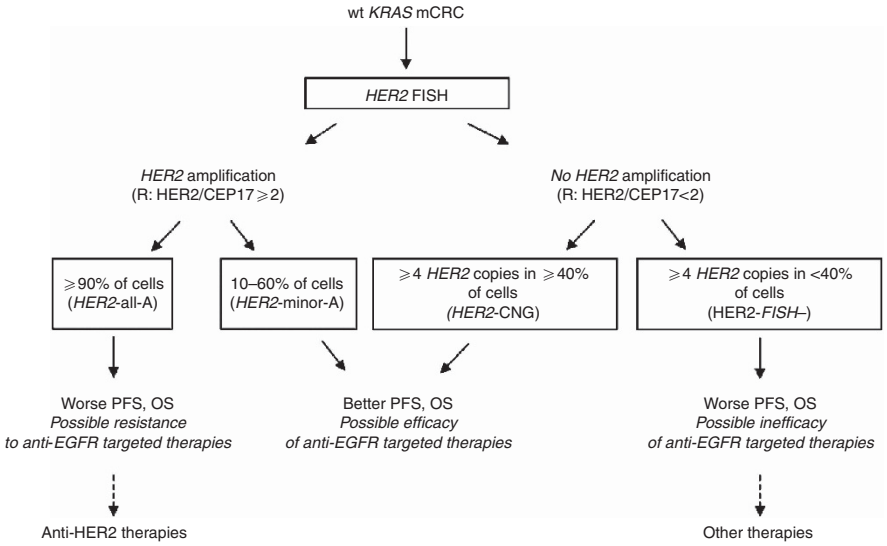

Figure 6. Practical interpretation chart to evaluate *HER2* FISH profile and potential clinical implications depending on *HER2* gene copy number status. mCRC = metastatic colorectal cancer; OS = overall survival; PFS = progression-free survival; wt = wild type.

HER2 stimulus), bypassing the EGFR blockade with anti-EGFR MoAbs; thus also indicating that in these patients HER2 probably represents the more important pathway with respect to EGFR, both hierarchically and biologically. By contrast, tumours with *HER2* amplification in a minority of the cells or with *HER2* CNG related to chromosome 7 polysomy have a different pathogenesis, and therefore their growth is probably dependent upon disruptions in other genes or other pathways. In these tumours, *HER2* deregulation could result from chromosome instability, and *HER2* polysomy is probably the mirror of a general polyploid karyotype, as demonstrated in mCRC using conventional cytogenetic techniques in the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (database online, http://cgap.nci.nih.gov/Chromosomes/Mitelman). In support of this hypothesis, we observed that nearly all of the patients with a *HER2* gene CNG (90%) had a concomitant gain of the *EGFR* gene (*HER2* FISH + /*EGFR* FISH +). Therefore, *HER2* FISH + * patients might be more likely to respond to anti-EGFR drugs for reasons not strictly associated with the *HER2* gene itself but rather related to the *EGFR* gene or to a complex karyotype.

On the other hand, the general finding that the oncogene deregulation can be linked to discordant disease behaviour depending on the proportion of cells that carry the alteration is not peculiar of *HER2*, as it has also been proposed for *EGFR*. Indeed, in non-small cell lung cancers treated with gefitinib or erlotinib, sensitive patients have the *EGFR* T790M mutation in only a few cells, whereas resistant patients exhibit *EGFR* T790M in the majority of the tumour cells (Ma *et al*, 2011; Benlloch *et al*, 2012).

The biological reason to explain the inefficacy of EGFR-targeted therapies in *HER2*-FISH − tumours could again be related both to kariotypic features (that probably in this case is less heterogeneous and near-diplod) or to a tumour growth that is *EGFR*/*HER2*-independent, according to a recent paper (Li *et al*, 2011), thus suggesting that other treatment regimens should be used for these patients (Figure 6).

In conclusion, our results, which deserve a confirmation in larger (not only retrospective) studies, revealed that the response to anti-EGFR MoAbs may be influenced by the *HER2* gene copy number status (as detected by FISH) in *KRAS* wt mCRC patients. The presence of *HER2* gene amplification throughout the tumour (*HER2*-all-A patients) is associated not only with resistance to cetuximab and panitumumab but may also identify patients who could benefit from specific anti-HER2 drugs (i.e., trastuzumab) or from combined EGFR/HER2 targeted agents (i.e., lapatinib) (Figure 6). By contrast, *HER2*-CNG or amplification in a minority of tumour cells (*HER2* FISH + *) may be a useful tool to assign patients to anti-EGFR-targeted therapies (Figure 6), thus representing an alternative option to evaluate *EGFR* gene status by FISH, which suffers from several limitations despite being proposed as a good predictive marker (Sartore-Bianchi *et al*, 2012).

Because of the aforementioned clinical implications, consistent and accurate *HER2* gene copy number assessment appears to be relevant for establishing the correct therapeutic regimen in mCRC patients.

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