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Brief Description of Novelty and Impact
Monoclonal antibodies targeting EGFR are effective only in a small proportion of colorectal cancer patients. Individuals with KRAS mutant tumours do not benefit from these agents and are excluded from treatment by current prescription guidelines. Here we show that amplification of the KRAS oncogene occurs in a small fraction of KRAS wild-type cases and it is causally associated with resistance to anti-EGFR treatment in colorectal cancer cells and patients.

Potential Conflicts of interest
Dr Pierre Laurent-Puig has received honoraria from Amgen and Merck-Serono, the manufacturers of panitumumab and cetuximab, respectively.

Abstract

KRAS mutations are the most common oncogenic event in colorectal (CRC) cancer progression and their occurrence is associated with lack of response to anti EGFR targeted therapies. Using preclinical models and patients' samples we recently reported that the emergence of KRAS mutations but also KRAS amplification is associated with acquired resistance to the EGFR inhibitors cetuximab or panitumumab. We reasoned that KRAS amplification may also be responsible for primary resistance to these agents. Furthermore, while the prevalence of KRAS mutations has been well established in CRC, little is known about the frequency of KRAS amplification in large CRC series. We performed a screening of 1039 CRC samples to assess the prevalence of KRAS amplification in this tumour type and further evaluated the role of this genetic alteration on the sensitivity to anti EGFR therapies. We detected KRAS amplification in 7/1039 (0.67%) and 1/102 evaluable CRC specimens and cell lines, respectively. KRAS amplification was mutually exclusive with KRAS mutations. Tumours or cell lines harbouring this genetic lesion are not responsive to anti-EGFR inhibitors. Although KRAS amplification is an infrequent event in CRC, it might be responsible for precluding response to anti-EGFR treatment in a small proportion of patients.

Introduction

Patients affected by metastatic colorectal cancer (mCRC), the most aggressive stage of this neoplastic disease, have a 5-year survival rate of less than 5%. In this setting, therapeutic options include monoclonal antibodies (MoAbs) targeting the Epidermal Growth Factor Receptor (EGFR), such as cetuximab and panitumumab.[1] Both molecules bind to the extracellular domain of the EGFR thus leading to inhibition of its downstream signalling. Anti-EGFR monoclonal antibodies are effective only in a small subset (10%) of mCRC patients.[2] The reasons for such a limited success rate prompted intense investigations. Indeed, we and others found that genetic alterations in oncoproteins modulating EGFR signalling (KRAS, BRAF, PIK3CA, PTEN and, recently, HER2, are key determinants of 'primary' resistance to anti EGFR therapies.[3-7] While all the above mentioned genetic alterations account for approximately 60-70% anti-EGFR resistant cases, the list of resistance biomarkers is still incomplete.

Nearly all patients who achieve clinical response to anti EGFR therapies show disease progression after 3-12 months.[3] The reasons why response is relatively short-lived and tumours become refractory to further anti *EGFR* treatment have just began to be elucidated. [8] While we have recently found that selection/acquisition of KRAS mutations occurred in a significant proportion of samples from patients who progressed on cetuximab, KRAS amplification was causally responsible for acquired resistance to anti-EGFR treatment in one CRC patient.[9]

Recent reports indicate the occurrence of KRAS amplification in a small percentage of several different solid malignancies, including NSCLC, head and neck SCC, ovarian and colorectal cancer.[10-18]

The purpose of this study was to determine the prevalence of KRAS gene amplification in a large dataset of CRC samples and assess the possible predictive role of KRAS gene copy number status in response to anti-EGFR treatment in mCRC.

Materials and methods

KRAS Immunohistochemistry.

To identify the best routine procedure for KRAS immunostaining, we preliminarily tested different dilutions of two different available antibodies (Santa-Cruz clone F234, cat. sc-30 and Abnova clone 3B10-2F2, cat. H00003845-M01) in an initial training set of 166 samples, for which we also determined KRAS status by FISH analysis and real time gene copy number. From these tests, all positive cases with both FISH and real time gene copy number were strongly positive also by IHC. No staining was observed in cases negative by FISH and real time gene copy number. Following optimization, for all tumours KRAS protein expression was evaluated by immunohistochemistry performed on 3 µm thick tissue sections using a 1:100 dilution of the specific KRAS (F234) antibody (SC-30, mouse monoclonal IgG2a Santa Cruz Biotechnology) and the automated system BenchMark Ultra (Ventana Medical System, Inc., Roche), as previously described.[9]

KRAS Fluorescent in situ hybridisation (FISH) analysis.

Dual colour FISH analysis was performed as previously described,[9] using for each slide a 10 µl mix- probe made up by 1 µl CEP12 alpha satellite probe (12p11-q11) labelled in SpectrumOrange (Vysis, Downers Grove, IL. USA), 1 µl BAC (Bacterial Artificial Chromosome) genomic probe RP11-707G18 (12p12.1) spanning an approximately 176 kb region encompassing the KRAS gene, labelled in SpectrumGreen (Bluegnome) and 8 µl LSI-WCP hybridisation buffer (Vysis, Downers Grove, IL. USA). Samples with a ratio greater than 3 between KRAS gene and chromosome 12 centromere signals, in at least 10% of 100

cells analysed in 10 different fields, were scored as positive for KRAS gene amplification. Healthy tissue was identified by two expert histopathologists (S.V. and M.G.) after microscopic visualization of slides stained by hematoxylin and eosin. Non-neoplastic colon mucosa adjacent to cancerous tissue was considered 'healthy' tissue and was used as internal negative control.

Tissue procurement

Tumour specimens were obtained through protocols approved by the Institutional Review Board of Ospedale Niguarda Ca' Granda (Milan, Italy, protocols 1014/09 and 194/2010), Digestive Oncology Unit at University Hospital Gasthuisberg (Leuven, Belgium), Regina Elena Cancer Institute (Rome, Italy), Dupuytre University Hospital Center (Limoges, France), Inserm UMR-S775 (Paris, France) and the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands). All tumour samples were formalin fixed paraffin embedded (FFPE). Sample collection included 559 specimens spotted on tissue microarrays obtained from patients enrolled in the CAIRO-2 phase III clinical trial.[19] Individual tissue slides from paraffin blocks were analysed for the remainder 480 cases. Overall, the tumour collection consisted of 150 primary CRC and 889 metastatic CRC samples. All patients provided informed consent and samples were procured and the study was conducted under the approval of the Review Boards and Ethical Committees of the Institutions.

Patient population and treatment regimens.

We retrospectively analysed 97 patients with histologically confirmed mCRC either at Ospedale Niguarda Ca' Granda (Milan, Italy) or at the Digestive Oncology Unit, University Hospital Gasthuisberg (Leuven, Belgium). Patients evaluated in this study were selected based on evidence that treatment outcome could be attributable to administration of either panitumumab or cetuximab (synergy with irinotecan should be taken into account for those

patients treated with cetuximab in combination with irinotecan in the chemorefractory setting). Patients were enrolled in clinical trials or received panitumumab or cetuximab *as per* label indication. For those patients who were treated with cetuximab in combination with irinotecan, refractoriness to irinotecan-based regimens was documented as disease progression during, or within, 6 months of receiving treatment. To avoid the confounding effect on response/resistance due to the concomitant administration of chemotherapy and bevacizumab in association with cetuximab, we have elected to exclude from the association analysis between response and KRAS status all those 277 patients who were randomized to the CAPOX + bevacizumab + cetuximab arm of the CAIRO-2 phase III first-line trial. Besides the above-mentioned inclusion criteria, the availability of tumour sample qualitatively and quantitatively suitable for molecular analyses was also a requirement for being considered in the present study. Clinical response was assessed every 6-8 weeks with radiological examination (computerized tomodensitometry or magnetic resonance imaging). The Response Evaluation Criteria in Solid Tumors (RECIST) [20] were adopted for evaluation and objective tumour response was classified into partial response (PR), stable disease (SD) and progressive disease (PD). Patients with SD or PD were defined as non-responders.[20] Two independent oncologists and radiologists verified in a blinded manner the clinical response for all patients.

Cell lines and drug viability assays

The LIM1215 and LIM2099 cell lines had been described previously,[21] and were obtained from Prof Robert Whitehead, Vanderbilt University, Nashville, with permission from the Ludwig Institute for Cancer Research, Melbourne branch, Australia. The LS513 and RCM1 cell lines were obtained from ATCC (LGC Standards S.r.l, Milan, Italy) and HSRRB (Osaka, Japan) repositories, respectively. The NCI-H630 cell line was purchased from the Korean Cell Bank (Seoul, Korea). The genetic identity of the cell lines used in this study was

confirmed by STR profiling (Cell ID, Promega). A lentiviral vector encoding for KRAS wild-type was employed to stably transduce LIM1215 cells, following previously described standard infection procedures.[22] Silencing of KRAS expression was achieved by reverse transfecting NCI-H630 cells with 25 nM KRAS or scramble (non-targeting) siRNA smartpools (Dharmacon) using DharmaFECT1 transfection reagent (Dharmacon). Cell lines were assayed at 120 h post transfection. Cetuximab was obtained from Pharmacy at Niguarda Ca' Granda Hospital, Milan, Italy. Cell lines were seeded in 100µl RPMI-1640 medium in 96-well plastic tissue culture plates. After serial dilutions, the monoclonal antibody cetuximab was added to cells and medium-only containing wells were added as controls. Plates were incubated at 37°C in 5% CO_2 for 5-6 days, after which cell viability was assessed by ATP content using the CellTiter-Glo® Luminescent Assay (Promega Madison, WI, USA).

Statistical analysis.

The objective tumour response was the endpoint of our exploratory study. Qualitative comparison of objective response to therapy (responders [PR] vs non-responders [PD + SD]) and KRAS gene amplification as a predictor was performed by the two-tailed Fisher's exact test to check possible significance. For in vitro experiments the comparison of cell viability in the presence of cetuximab was performed by One-way ANOVA with Bonferroni's multiple comparison test. The level of significance was set at $P<0.05$.

Results

Different techniques have been described in other studies to measure KRAS gene copy number status, including qPCR, deep sequencing, SNP arrays, FISH and CISH. In order to analyse a large number of samples we initially established a sensitive and convenient immunohistochemical (IHC) technique to rapidly detect KRAS overexpression in archived

FFPE CRC samples. KRAS protein expression was detected at cytoplasmic and membrane level. Samples were considered positive when the expression of protein was present in at least 10% of cells. Healthy tissue, i.e, normal colon mucosa, was used as internal negative control (Fig. 1*a*). Similarly to what is routinely performed for other markers, including HER2, only slides that were judged as stained strongly positive (IHC 2+ or 3+) by two independent pathologists were subsequently analysed by FISH.

In total, we screened 1039 CRC samples from five different institutions. Overall, seven of these samples were confirmed to carry KRAS gene amplification when analysed by FISH (Fig. 1*b*). We found that KRAS amplification in CRC is an infrequent event, with an overall prevalence of 0.67% (Fig. 1*c*). We reasoned that if KRAS amplification were functionally equivalent to the presence of KRAS mutations, the two alterations might occur in a mutual exclusivity fashion. Indeed, mutational status was available for 899 samples, of which 317 (35%) were found to be KRAS mutated but negative for the amplification (Fig. 1*d*).Additionally, none of the seven samples displaying KRAS amplification were found to harbour KRAS or BRAF mutations ($p = 0.0562$, two-tailed Fisher's exact test).

A proportion of samples analysed for this study was obtained from patients who were subsequently treated with panitumumab or cetuximab containing regimens (n=374). Of these, 277 individuals were randomized to the CAPOX + bevacizumab + cetuximab arm of the CAIRO-2 phase III first-line trial.[19] To avoid the confounding effect on response/resistance due to the concomitant administration of other drugs in association with cetuximab, these samples were excluded from the analysis of association between KRAS amplification and response. The remainder 97 patients with available clinical follow-up had KRAS wild-type tumours and were selected based on evidence that treatment outcome could be attributable to administration of either panitumumab or cetuximab. In this cohort,

the presence of KRAS amplification is suggested to be inversely associated with response to therapy. None of 44 KRAS wild-type patients who experienced a response (PR) to therapy displayed KRAS amplification, whereas 4/53 (7.5%) non-responder (NR) patients carried this genetic alteration (Fig. 1*e*; P=0.1237, NS, two tailed Fisher's exact test). These four KRAS amplified cases were found to be wild-type not only for KRAS (exons 2, 3 and 4), but also for NRAS (exons 2 and 3), BRAF (exon 15), PIK3CA (exons 9 and 20), and stained HER2 negative. This data indicates that occurrence of KRAS amplification might negatively interfere with the clinical response to monoclonal antibodies targeting EGFR.

To corroborate the above described clinical findings, we decided to assess whether KRAS amplification was associated with resistance to anti EGFR therapies also in preclinical models. We profiled a large dataset of CRC cell lines and found that only one out of 102 lines (1%) harboured KRAS amplification (Fig. 2*a*) with corresponding protein overexpression (Fig. 2*b*). We established that the KRAS amplified NCI-H630 cell line was wild type for KRAS, BRAF, NRAS and was intrinsically resistant to cetuximab similarly to KRAS mutant CRC models (Fig. 2*c*).[23] To prove that KRAS amplification was causally related to resistance to EGFR targeted monoclonal antibodies, we silenced KRAS expression in this line (Fig 2*d*). Down-regulation of KRAS expression was able to restore sensitivity to cetuximab in NCI-H630 cells, thus unequivocally establishing the role of KRAS amplification in conferring resistance to anti-EGFR therapies in this model (Fig 2*e*).

To further assess the functional role of KRAS amplification, we used forward genetics to over-express KRAS in a CRC cell line sensitive to EGFR targeted monoclonal antibodies. To this aim, we employed the EGFR expressing and cetuximab sensitive LIM1215 cell line (Fig. 2*c*), which was previously reported to be wild-type for KRAS, BRAF, PIK3CA and PTEN.[23] LIM1215 cells were transduced with serial dilutions of lentiviral particles containing

a vector encoding for KRAS wild-type cDNA (Fig. 2*f*) and treated with cetuximab at a clinically relevant dose of 300 nM, corresponding to the serum $C_{through}$ concentration of cetuximab over several weeks' treatment.[24] Cells expressing lower KRAS levels (dilutions 1:8 and 1:10) were still sensitive to EGFR inhibition, while cells displaying more abundant KRAS (virus titres 1:2 and 1:4) were significantly more resistant to cetuximab than control cells (Fig. 2*g*).

Discussion

We found that KRAS amplification in CRC is an infrequent event, with an overall prevalence of 0.67% (7/1039 cases). So far, this is the largest study to analyse KRAS amplification in CRC. Indeed, a previous report employed real-time quantitative PCR and identified increased KRAS gene copy number in 2 of the 96 tumour pairs analysed (2.1%), of which one with 4 and the other with 27 copies of the KRAS gene.[12] It is possible that the relatively lower prevalence of KRAS amplification in our study reflects the different methodology employed to detect gene copy number status. In order to rule out this possibility we applied real time quantitative PCR on a selection of 166 CRC samples, for which sufficient archived material was available for DNA extraction. The same three samples identified as KRAS amplified by this technique were also scored positive by immunostaining and FISH analyses, thus cross-validating these three different analytical methods (data not shown).

Our analyses suggest a mutual exclusivity pattern between KRAS amplification and KRAS (or BRAF) mutations, but this trend should be further validated in larger cohorts. It is intriguing to observe that gene expression changes induced by over-expression of human KRAS wild-type in NIH-3T3 murine cells were reported to resemble those induced by

oncogenic (mutant) KRAS variants.[12] Given that KRAS hyper-activation has been previously associated with induction of senescence,[25] it is tempting to speculate that amplification of KRAS mutant alleles – but not of KRAS wild-type – is counter selected during tumour progression. In this regard, we were able to detect KRAS amplification in all tissue samples (from three distinct individuals) for which both primary CRC and metastatic specimens were available, suggesting that this genetic alteration is conserved during tumour progression. Future studies including adenoma series will be needed to assess at which stage of oncogenesis KRAS amplification occurs.

The presence of KRAS mutations is the only validated marker to predict lack of clinical benefit in mCRC patients treated with anti-EGFR monoclonal antibodies. Genetic alterations in other oncogenes modulating EGFR signalling (mutations in BRAF or NRAS, and, recently, HER2 amplification), are also key determinants of primary resistance to anti EGFR therapies [3, 5-7] and represent promising additional markers. However, KRAS and all of the above mentioned genetic alterations account for approximately 60-70% anti-EGFR resistant cases, and the list of resistance biomarkers is still incomplete. We therefore hypothesized that increased KRAS gene copy number could be responsible for refractoriness to EGFR therapies in a small proportion of cases.

We detected KRAS amplification in 4/97 cases selected among tumours wild-type for KRAS from patients who had received either cetuximab or panitumumab. It is remarkable that all four KRAS amplified cases were found among the 53 non-responding patients, while none of the 44 responders had tumours carrying this molecular alteration. The low prevalence of this genetic lesion prevents us from establishing a statistical inverse association between KRAS amplification and response to anti-EGFR therapies; and it is

expected that over a thousand samples with clinical follow-up and annotated KRAS status will be needed to address this question.

To substantiate clinical findings, we used preclinical models of CRC to assess at the molecular level whether and how KRAS amplification could affect response to anti-EGFR therapies. We found that the occurrence of KRAS amplification or the exogenous over-expression of KRAS wild-type in otherwise sensitive cells dramatically impairs their response to cetuximab. Importantly, silencing of KRAS is able to restore sensitivity to cetuximab in a CRC cell line carrying endogenous KRAS amplification. These results have two relevant implications. Firstly, they show that the presence of KRAS amplification directly affects response to EGFR targeted agents at the cellular level. Secondly, they suggest that the correlation between the occurrence of KRAS amplification and the ineffective clinical response to cetuximab or panitumumab is not generically due to the poorer prognosis of these tumours.

Although the clinical implications of KRAS amplification requires further validation as a predictive biomarker of primary resistance to anti-EGFR therapy, we have recently reported development of KRAS amplification in one patient with acquired resistance to cetuximab. [9] Altogether, preclinical and clinical evidence points to KRAS amplification as a possible novel mechanism of resistance to EGFR targeted therapies in CRC.

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Figure Legends

Figure 1. Representative colorectal tumour sections showing KRAS immunostaining (*a*) and gene copy number status by FISH (*b*) in KRAS amplified CRC samples. Dual colour FISH analysis was performed using a CEP12 alpha satellite probe (12p11-q11) labelled in SpectrumOrange and a BAC (Bacterial Artificial Chromosome) genomic probe RP11-707G18 (12p12.1) spanning an approximately 176 kb region encompassing the KRAS gene, labelled in SpectrumGreen. Prevalence of KRAS amplification (*c*) in 1039 unselected CRC samples; and (*d*) in a subgroup with known KRAS molecular status. (*e*) Clinical response to treatment with either panitumumab of cetuximab in 97 mCRC patients with known KRAS gene copy number status.

Figure 2. (*a*) The NCI-H630 CRC cell line displays KRAS amplification (as double minutes), as confirmed by FISH analysis. KRAS locus BAC DNA (probe RP11-707G18; green) and chromosome 12 CEP12 alpha satellite probe (12p11-q11; red) were hybridized to the metaphase spreads of NCI-H630 cells. (*b*) Biochemical analysis of KRAS expression in a panel of CRC cell lines. KRAS is overexpressed in NCI-H630 cells. Actin was used as a loading control (*c*) Colorectal cancer cell lines of the indicated KRAS genotype were treated for 6 days with increasing concentrations of cetuximab. Cell viability was assayed by the ATP assay. Data points represent means ± SD of three independent experiments. (*d*) KRAS expression was knocked down by siRNA in the NCI-H630 line. Cells not transfected are labelled as ‘nt’, while ‘scramble’ indicates cells transfected with non-targeting siRNA pool. Actin was used as a loading control. (*e*) KRAS silencing restores sensitivity to cetuximab in the KRAS amplified NCI-H630 cell line. Results are plotted as means± SEM of triplicate observations. (*f*) LIM1215 cells were transduced with scalar dilutions of lentiviral particles containing a vector encoding for KRAS wild-type cDNA or a control empty vector. Western

Blot shows level of ectopic KRAS expression in transduced cells; actin was used as a loading control. The amount of endogenous KRAS protein in the KRAS amplified NCI-H630 cell line is shown as reference. (*g*) Overexpression of KRAS wild-type confers resistance to cetuximab in LIM1215 sensitive colorectal cancer cells. Cells were exposed to the drug at 300 nM for 6 days, after which cell viability was measured by ATP content. Data points represent means ± SEM of triplicate observations from a representative experiment. **P<0.01 and ***P<0.001 by One-way ANOVA with Bonferroni's multiple comparison test.

Figure 1

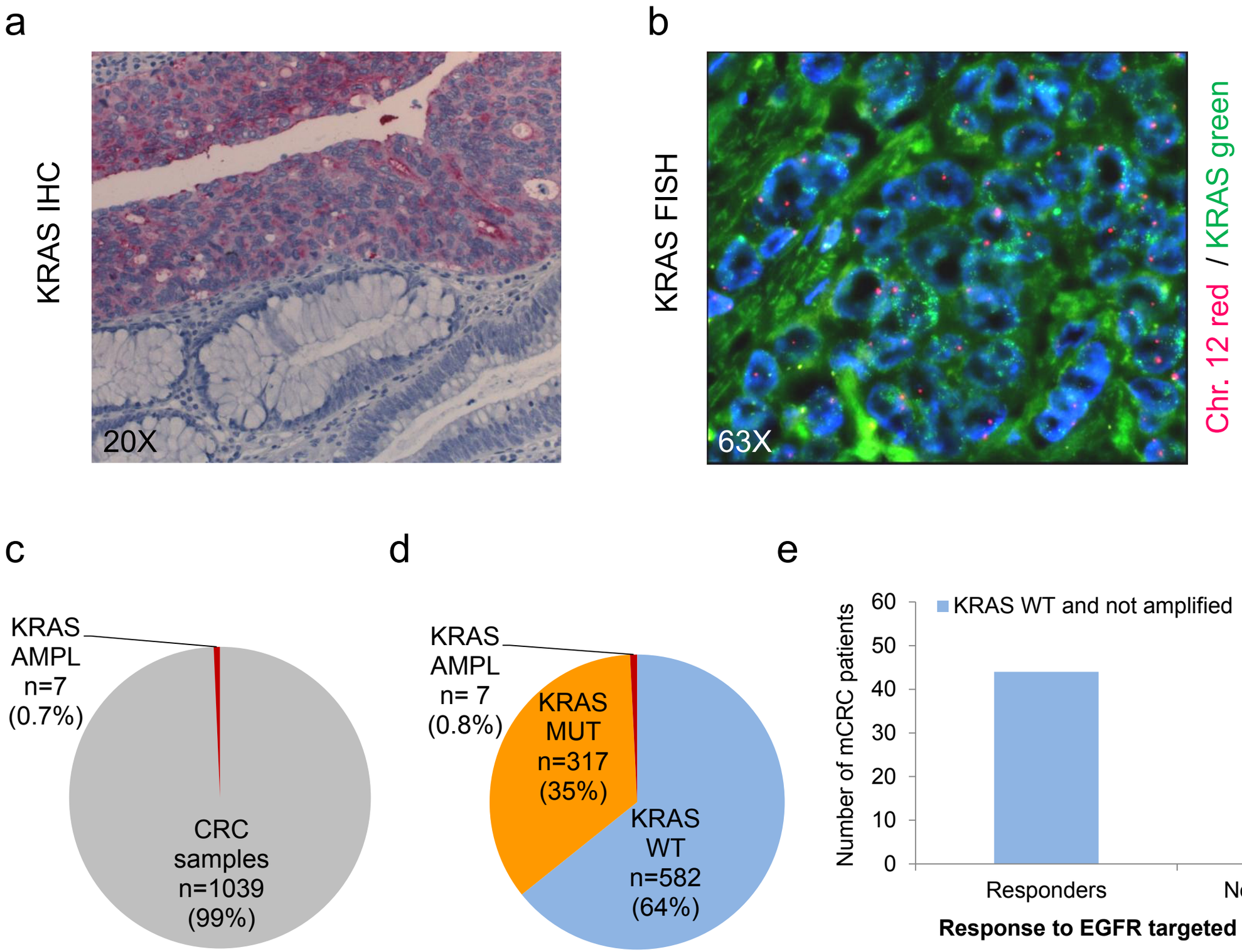
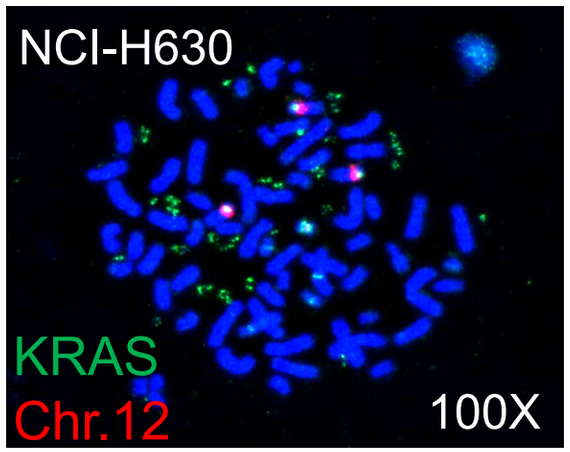
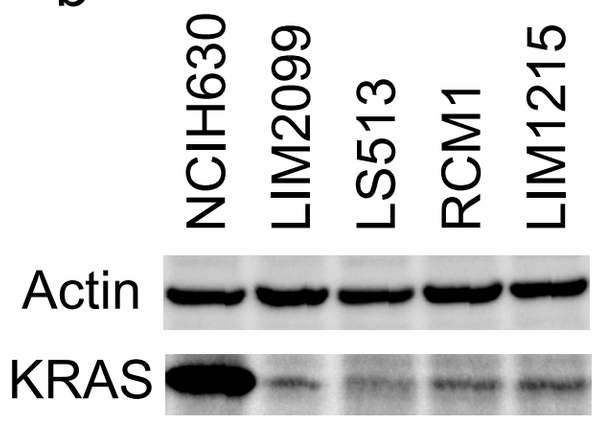

a
KRAS IHC
20X
b
KRAS FISH
63X
Chr. 12 red / KRAS green
c
KRAS AMPL n=7 (0.7%)
CRC samples n=1039 (99%)
d
KRAS AMPL n= 7 (0.8%)
KRAS MUT n=317 (35%)
KRAS WT n=582 (64%)
e
KRAS WT and not amplified
KRAS WT amplified
Number of mCRC patients
60
50
40
30
20
10
0
4
Responders
Non Responders
Response to EGFR targeted monoclonal Ab

Figure 2

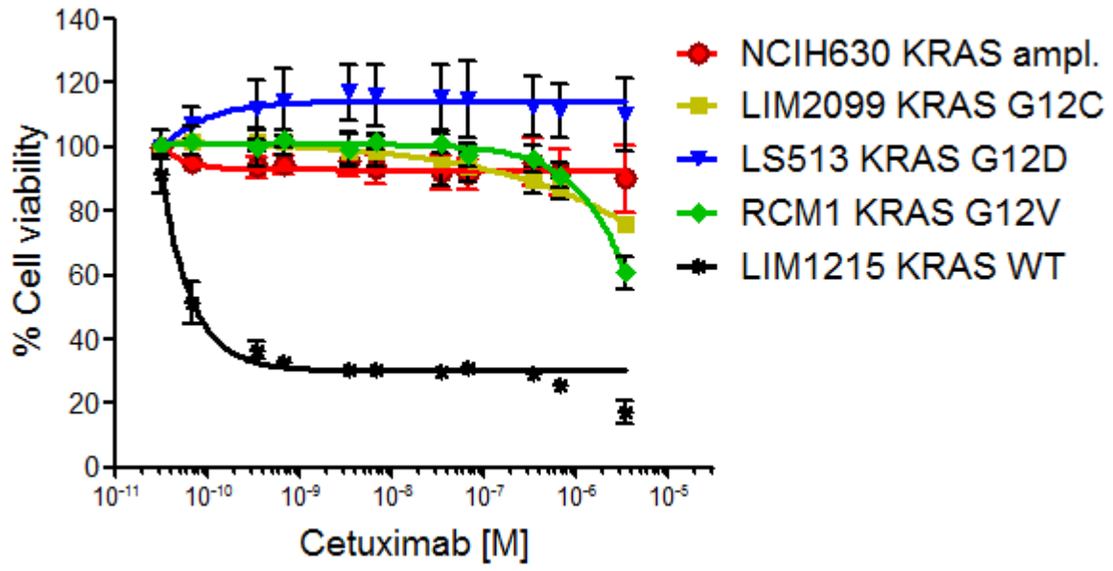
a



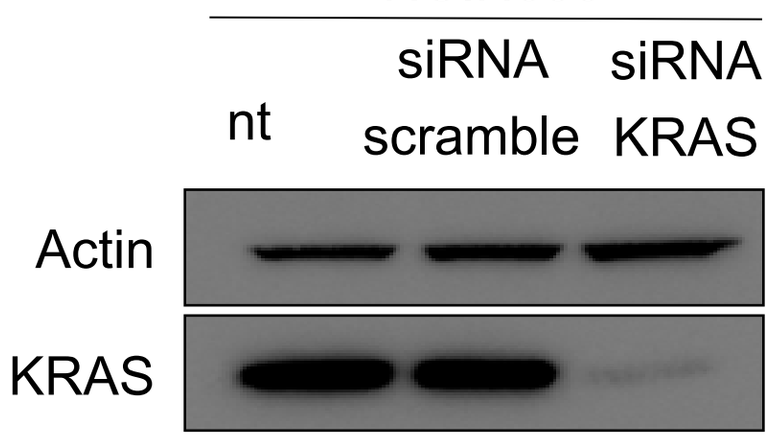
b



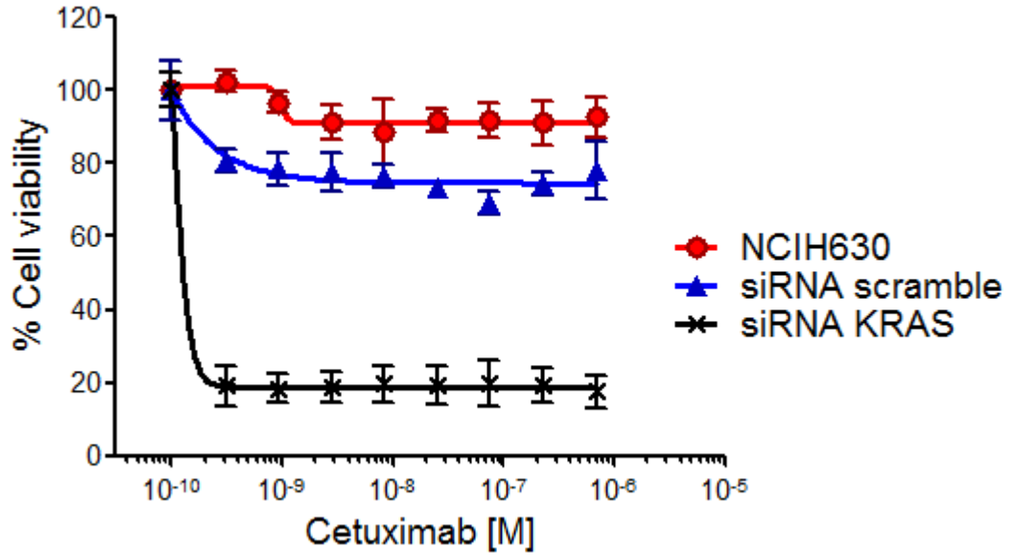
c



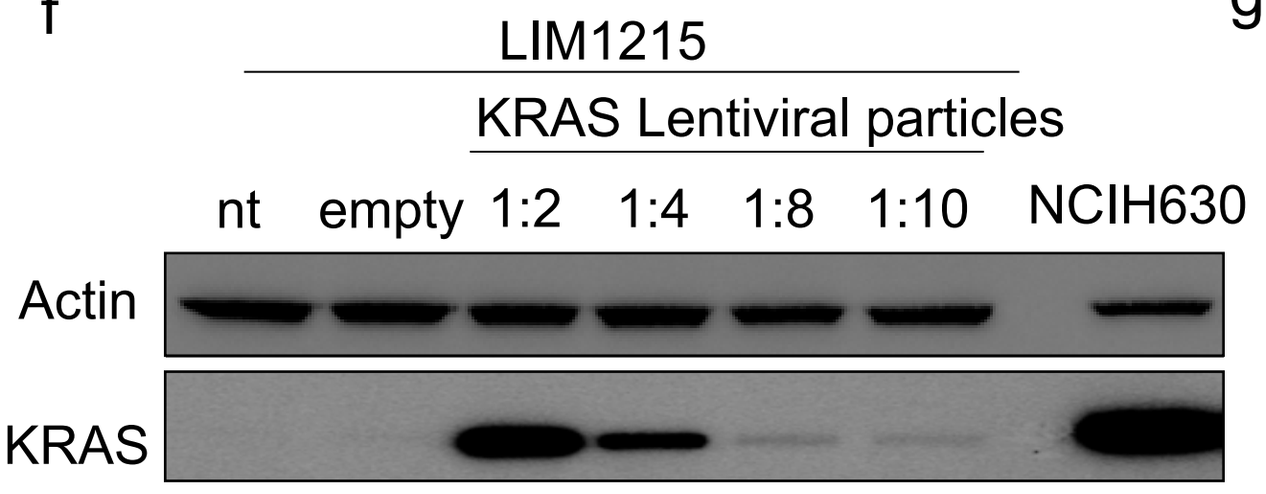
d



e



f



g