Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study

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Summary

Background **The antiepidermal growth factor receptor (antiEGFR) monoclonal antibodies cetuximab and panitumumab have good clinical activity in about 10% of patients with metastatic colorectal cancer that is resistant to chemotherapy. The molecular mechanisms underlying clinical response or resistance to these agents are unknown.**

Methods **Tumours from 31 patients with metastatic colorectal cancer who had either an objective response (n=10) or stable disease or progressive disease (n=21) after treatment with cetuximab or panitumumab were screened for genetic changes in *EGFR* or its immediate intracellular effectors. Specifically, we assessed the *EGFR* copy number and the mutation profile of the *EGFR* catalytic domain and of selected exons in *KRAS*, *BRAF*, and *PIK3CA*.**

Results **Eight of nine of patients with objective responses who were assessable by fluorescence in-situ hybridisation (FISH) had an increased *EGFR* copy number. By contrast, one of 21 non-responders assessable by FISH had an increased *EGFR* copy number ($p<0·0001$ for responders *vs* non-responders, Fisher's exact test). The mutation status of the *EGFR* catalytic domain and its immediate downstream effectors *PIK3CA*, *KRAS*, and *BRAF* did not correlate with disease response. In colorectal-cancer cell lines, the concentration of cetuximab that completely inhibited proliferation of cells with amplified *EGFR* copy number did not affect proliferation of cells with unamplified *EGFR*.**

Interpretation **We propose that the response to antiEGFR treatment has a genetic basis and suggest that patients might be selected for treatment on the basis of *EGFR* copy number.**

Introduction

The therapeutic options against metastatic colorectal cancer, the third most common cause of death from cancer, have been expanded with the introduction of monoclonal antibodies against the extracellular domain of epidermal growth factor receptor (EGFR).[1] The chimeric IgG1 antibody cetuximab and the fully human IgG2 antibody panitumumab both have good clinical activity in about 10% of patients with chemotherapy-resistant metastatic colorectal cancer,[1–4] but the molecular mechanisms underlying clinical response or resistance are unknown. Neither the diagnostic characteristics nor the degree of EGFR expression in tumour tissue (as confirmed by immunohistochemical analyses) correlate with clinical response.[2–4] An understanding of the molecular basis for clinical sensitivity or resistance to antiEGFR agents might identify patients who are likely to benefit from treatment with cetuximab or panitumumab.

The biology of EGFR has been studied in detail by use of genetic and biochemical approaches.[5,6] When a ligand binds to the extracellular part of the receptor, the receptor dimerises and its enzymatic activity is activated, resulting in phosphorylation of the intracellular domain. Subsequently, cellular effectors bind to phosphorylated residues of the intracellular domain and are activated, mainly through relocalisation to the plasma membrane. The small G protein RAS, the protein kinase RAF, and phosphoinositide 3-kinase have central roles as intracellular mediators of EGFR signalling. Mutations in *EGFR* and its effectors have been found in various cancers.[7,8] We postulated that the clinical response to cetuximab or panitumumab was associated with molecular changes affecting EGFR or its immediate intracellular signal transducers.

Methods

Patients

We assessed 31 patients enrolled into clinical trials of panitumumab (Amgen, Thousand Oaks, CA, USA) or cetuximab (Erbitux®, Merck, Milan, Italy) for treatment of EGFR-expressing metastatic colorectal cancer at Ospedale Niguarda Ca' Granda, Milan, Italy. Tumour sensitivity or resistance to these monoclonal antibodies was confirmed radiologically in all patients (table 1). Patients were selected on the basis of the availability of sufficient tumour tissue. All patients had EGFR-expressing metastatic colorectal cancer and 1% or more malignant cells that stained for EGFR on immunohistochemical analysis with DAKO EGFRPharmDX kit (DakoCytomation, Glostrup, Denmark) done in the central laboratory of every clinical trial.[3]

Because cetuximab and panitumumab both target the ligand-binding domain of EGFR,[5,9,10] they are thought to have similar clinical activity, although fewer infusion reactions have been noted with panitumumab[4] than with cetuximab. Therefore, our analyses combined patients

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Patient number	Sex	Age (years)	Performance status*	Previous chemotherapy regimens for metastatic disease	Regimens for metastatic disease	AntiEGFR treatment	Tumour response		Molecular analysis of *EGFR*	
							Best response	Duration (weeks)	Copy number	Sequence†
1	F	52	0	3	Fluorouracil and folinic acid; FOLFOX; irinotecan	Cetuximab and chemotherapy‡	PR	48	Increased	Wildtype
2	M	59	0	3	Fluorouracil and folinic acid; FOLFOX; FOLFIRI	Cetuximab and chemotherapy‡	PR	36	Increased	Wildtype
3	M	69	0	2	FOLFOX; FOLFIRI	Cetuximab and chemotherapy‡	PR	>36§	Increased	Wildtype
4	M	57	1	3	Fluorouracil and folinic acid; FOLFOX; FOLFIRI	Cetuximab	PR	30	Inconclusive	Wildtype
5	M	59	0	3	FOLFOX; capecitabine; FOLFIRI	Panitumumab	PR	33	Increased	Wildtype
6	F	62	1	2	FOLFOX; FOLFIRI	Panitumumab	PR	24	Increased	Wildtype
7	M	57	1	2	FOLFIRI; FOLFOX	Panitumumab	PR	16	Increased	Wildtype
8	M	79	1	0	NA	Cetuximab	PR	>16§	Normal	Wildtype
9	F	78	1	3	FOLFOX; capecitabine; FOLFIRI	Panitumumab	PR	>12§	Increased	Wildtype
10	M	85	1	0	NA	Cetuximab	PR	>8§	Increased	Wildtype
11	M	60	0	1	Irinotecan	Cetuximab and chemotherapy‡	SD	30	Normal	Wildtype
12	M	59	0	2	FOLFOX and FOLFIRI	Cetuximab and chemotherapy‡	SD	24	Normal	Wildtype
13	M	75	0	2	FOLFOX; FOLFIRI	Cetuximab and chemotherapy‡	SD	24	Normal	Exon 21 Gly857Arg
14	F	80	1	0	NA	Cetuximab	SD	>36§	Normal	Wildtype
15	F	52	0	4	FOLFOX; irinotecan; capecitabine; FOLFIRI	Panitumumab	SD	32	Inconclusive	Wildtype
16	M	71	1	2	FOLFOX; FOLFIRI	Panitumumab	SD	>16§	Normal	Wildtype
17	M	58	0	0	NA	Cetuximab	PD	NA	Increased	Wildtype
18	M	54	1	2	FOLFOX; irinotecan and weekly high-dose fluorouracil and folinic acid	Cetuximab and chemotherapy‡	PD	NA	Normal	Wildtype
19	M	62	1	0	NA	Cetuximab	PD	NA	Normal	Wildtype
20	M	74	0	3	Irinotecan and capecitabine; FOLFOX; FOLFIRI	Cetuximab and chemotherapy‡	PD	NA	Normal	Wildtype
21	M	41	0	3	FOLFOX; FOLFIRI; irinotecan	Cetuximab and chemotherapy‡	PD	NA	Normal	Wildtype
22	F	72	1	0	NA	Cetuximab	PD	NA	Normal	Wildtype
23	M	72	1	0	NA	Cetuximab	PD	NA	Normal	Wildtype
24	M	78	1	0	NA	Cetuximab	PD	NA	Normal	Wildtype
25	F	75	1	0	NA	Cetuximab	PD	NA	Normal	Wildtype
26	M	75	1	0	NA	Cetuximab	PD	NA	Normal	Wildtype
27	M	56	1	1	Oxaliplatin, irinotecan, fluorouracil, and folinic acid	Panitumumab	PD	NA	Normal	Wildtype
28	F	67	0	2	FOLFOX; FOLFIRI	Panitumumab	PD	NA	Normal	Wildtype
29	M	77	1	0	NA	Cetuximab	PD	NA	Normal	Wildtype
30	M	54	0	2	FOLFOX; FOLFIRI	Panitumumab	PD	NA	Normal	Wildtype
31	F	65	1	3	Fluorouracil and folinic acid; FOLFOX; FOLFIRI	Panitumumab	PD	NA	Normal	Wildtype

F=female. FOLFOX=oxaliplatin, fluorouracil, and folinic acid. PR=partial response. M=male. FOLFIRI=irinotecan, fluorouracil, and folinic acid. NA=not applicable. SD=stable disease. PD=progressive disease. *Eastern Cooperative Oncology Group performance status at time of starting monoclonal-antibody treatment. †Mutational status of *EGFR* exons 18, 19, and 21. ‡Irinotecan-based chemotherapy. §Maintained response as of February, 2005.

Table 1: **Clinical characteristics and *EGFR* molecular changes in tumours of patients with metastatic colorectal cancer**

treated with either monoclonal antibody. 12 patients received cetuximab monotherapy, nine received cetuximab plus irinotecan-based chemotherapy (Camptò®, Aventis, Milan, Italy), and ten received panitumumab monotherapy. Cetuximab alone was assigned either as first-line treatment in the EMR 202-600 phase II trial or as third-line treatment in the monotherapy group of the BOND (Bowel Oncology with Cetuximab Antibody) phase II trial[3] for patients refractory to irinotecan, and was administered as loading dose 400 mg/m^2 intravenously, followed by 250 mg/m^2 once a week until progression[3] Cetuximab plus irinotecan were assigned as third-line treatment in the combination group of the BOND phase II trial and in the MABEL (Monoclonal Antibody Erbitux in a European Pre-Licence Study) phase II trial for patients refractory to irinotecan. Cetuximab was given in the same dose and schedule as for the monotherapy group, and irinotecan was given in the same dose and schedule to which every patient had previously become resistant, both until disease progression. Refractoriness to irinotecan was defined as documented disease progression during, or within,

Panel: Primers used for mutation and gene-copy analyses

Mutation

EGFR

Exon 18
- Forward 5′-GCTGAGGTGACCCTTGTCTC-3′
- Reverse 5′-ACAGCTTGCAAGGACTCTGG-3′
- Sequencing 5′-TGGAGCCTCTTACACCCAGT-3′

Exon 19
- Forward 5′-CCCAGTGTCCCTCACCTTC-3′
- Reverse 5′-CCACACAGCAAAGCAGAAAC-3′
- Sequencing 5′-GCTGGTAACATCCACCCAGA-3′

Exon 21
- Forward 5′-TGATCTGTCCCTCACAGCAG-3′
- Reverse 5′-TCAGGAAAATGCTGGCTGAC-3′
- Sequencing 5′-TTCAGGGCATGAACTACTTGG-3′

KRAS

Exon 2
- Forward 5′-GGTGGAGTATTTGATAGTGTATTAACC-3′
- Reverse 5′-AGAATGGTCCTGCACCAGTAA-3′-
- Sequencing 5′-TCATTATTTTTATTATAAGGCCTGCTG-3′

PI3KCA

Exon 9
- Forward 5′-GGGAAAAATATGACAAAGAAAGC-3′
- Reverse 5′-CTGAGATCAGCCAAATTCAGTT-3′
- Sequencing 5′-TAGCTAGAGACAATGAATTAAGGGAAA-3′

Exon 20
- Forward 5′-CTCAATGATGCTTGGCTCTG-3′
- Reverse 5′-TGGAATCCAGAGTGAGCTTTC-3′
- Sequencing 5′-TTGATGACATTGCATACATTCG-3′

BRAF

Exon 15
- Forward 5′-TGCTTGCTCTGATAGGAAAATG-3′
- Reverse 5′-AGCATCTCAGGGCCAAAAAT-3′
- Sequencing 5′-TGTTTTCCTTTACTTACTACACCTCA-3′

Gene copy

EGFR

Non-repetitive region
- Forward 5′-GAATTCGGATGCAGAGCTTC-3′
- Reverse 5′-GACATGCTGCGGTGTTTTC-3′

Line-1
- Forward 5′-AAAGCCGCTCAACTACATGG-3′
- Reverse 5′-TGCTTTGAATGCGTCCCAGAG-3′

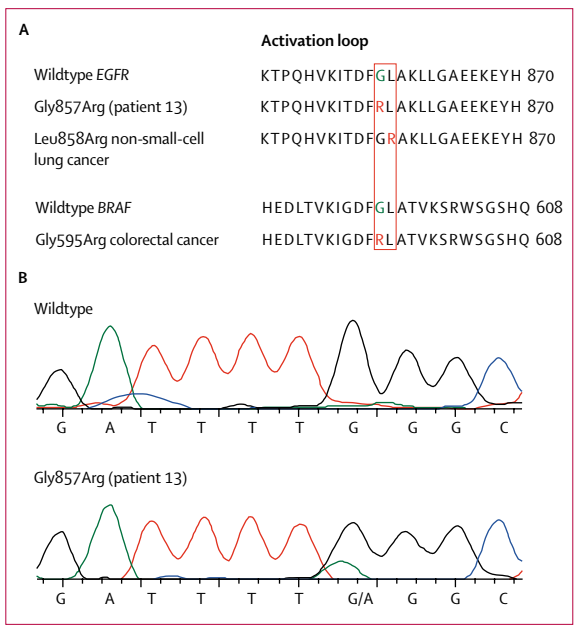


Figure 1: **Missense heterozygous mutation in exon 21 of *EGFR* (Gly857Arg) in tumour of patient 13**
The mutation affects a crucial residue in the activation loop of the EGFR kinase domain. This mutation is one aminoacid away from a Leu858Arg mutation found in gefitinib and erlotinib responders in non-small-cell lung cancer, and a mutation affecting an analogous residue in *BRAF* (Gly595Arg) reported in colorectal cancer.

3 months of receiving an irinotecan regimen. 6 mg/kg panitumumab given intravenously every 2 weeks until progression was allocated as third-line or fourth-line treatment for patients resistant to regimens consisting of oxaliplatin and irinotecan in the phase III ABX-EGF 20020408 and crossover ABX-EGF 20020194 trials. Treatment protocols were approved by the ethics committee of Ospedale Niguarda Ca' Granda, and patients gave written informed consent for *EGFR* analysis and for receiving the study treatment. Tumour response was assessed with CT or MRI by use of RECIST (Response Evaluation Criteria in Solid Tumors) criteria according to clinical protocols by radiologists at Niguarda Ca' Granda Hospital and by independent radiologists.

DNA extraction and mutation analyses

To identify the molecular basis for the response to panitumumab or cetuximab in metastatic colorectal cancer, we assessed the mutation status of the region corresponding to the catalytic domain of *EGFR* (ie, exons, 18, 19, and 21). DNA was extracted from paraffin-embedded samples of colorectal cancer. For every patient, 10-μm sections were prepared, and an additional representative 2-μm section was deparaffinised, stained with haematoxylin and eosin, and analysed for detailed morphology. Regions of tumour tissue were marked, and this tissue was extracted with 0·2 mol/L sodium hydroxide in 1 mmol/L edetic acid and neutralised with 100 mmol/L TRIS-TE (pH 6·5). After extraction, DNA was purified with Qiagen PCR purification kit (Qiagen, Milan, Italy). Exon-specific primers and sequencing primers for *EGFR* were designed by use of Primer3 software[11] and synthesised by Invitrogen™, Paisley, UK (panel).

We then assessed whether the mutation status of the genes for three intracellular effectors—*KRAS*, *BRAF*, and *PIK3CA*—correlated with clinical response to cetuximab or panitumumab. We analysed exons where mutations

Patient number	*EGFR* copy-number ratio		Mutation analyses						
	CEP7	Nucleus	*EGFR* exon 18	*EGFR* exon 19	*EGFR* exon 21	*KRAS* exon 2	*PIK3CA* exon-9	*PIK3CA* exon 20	*BRAF* exon 15
Responders									
1	3·37	7·90	WT	WT	WT	WT	Glu545Lys	WT	WT
2	2·28	5·70*	WT	WT	WT	WT	WT	WT	WT
3	1·42	7·10	WT	WT	WT	WT	WT	WT	WT
4	NE	NE	WT	WT	WT	WT	WT	WT	WT
5	2·50	4·80	WT	WT	WT	WT	WT	WT	WT
6	2·13	6·80	WT	WT	WT	Gly13Asp	WT	WT	WT
7	3·27	8·20	WT	WT	WT	Gly12Asp	WT	WT	WT
8	1·03	1·65	WT	WT	WT	WT	WT	WT	WT
9	1·19	3·38	WT	WT	WT	WT	WT	WT	WT
10	8·75†	35·00†	WT	WT	WT	WT	WT	WT	WT
Non-responders									
11	0·98	1·80	WT	WT	WT	WT	WT	WT	WT
12	1·05	1·90	WT	WT	WT	WT	WT	WT	WT
13	0·95	1·75	WT	WT	Gly857Arg	WT	WT	WT	NE
14	1·06	1·80	WT	WT	WT	WT	WT	WT	WT
15	NE	NE	WT	WT	WT	Gly13Asp	WT	WT	WT
16	1·04	1·88	WT	WT	WT	Gly12Val	WT	WT	WT
17	4·68‡	20·2‡	WT	WT	WT	WT	WT	His1047Arg	WT
18	1·04	2·00	WT	WT	WT	Gly13Asp	WT	WT	WT
19	0·70	1·72	WT	WT	WT	WT	WT	WT	WT
20	0·99	1·95	WT	WT	WT	Gly12Val	WT	WT	WT
21	0·95	2·00	WT	WT	WT	WT	WT	WT	WT
22	1·00	1·90	WT	WT	WT	Gly12Ser	WT	WT	WT
23	1·20	2·10	WT	WT	WT	WT	WT	WT	WT
24	1·16	1·98	WT	WT	WT	Gly12Asp	WT	WT	WT
25	0·90	1·75	WT	WT	WT	WT	WT	WT	WT
26	0·96	1·85	WT	WT	WT	Gly12Asp	WT	WT	WT
27	0·91	1·70	WT	WT	WT	WT	WT	WT	WT
28	1·02	2·00	WT	WT	WT	Gly13Asp	WT	WT	WT
29	1·00	2·05	WT	WT	WT	WT	WT	WT	WT
30	1·03	2·00	WT	WT	WT	WT	WT	WT	WT
31	1·18	2·10	WT	WT	WT	WT	WT	His1047Arg	Glu599Val

WT=wildtype. NE=Not evaluable. *Increased *EGFR* copy number found in primary colorectal tumour before monoclonal-antibody treatment and in liver metastasis at time of progressive disease after monoclonal-antibody treatment. †Focal clustered. ‡Clustered.

Table 2: **Molecular changes in tumours of patients with metastatic colorectal cancer**

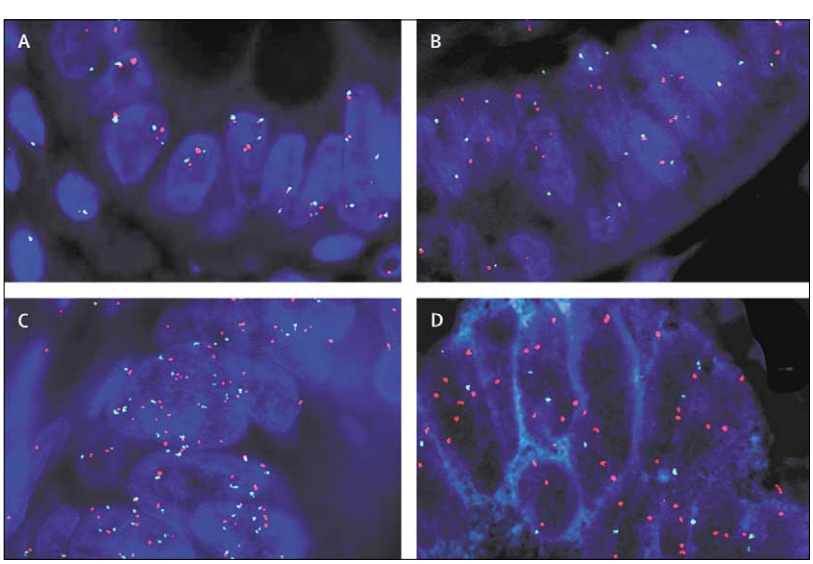


Figure 2: **Dual colour FISH assays for probes of *EGFR* (red) and chromosome seven (CEP7, green)**
(A) Balanced disomy in healthy colorectal mucosa. (B) Balanced disomy in tumour of patient 27. (C) Balanced polysomy in tumour of patient 3. (D) Amplification in tumour of patient 5.

occur with the highest frequencies in colorectal cancers (ie, *KRAS* exon 2, *BRAF* exon 15, and *PIK3CA* exons 9 and 20).[7] The nucleotide sequence corresponding to every exon was amplified from tumour-extracted genomic DNA and directly sequenced (panel).

Conditions for the amplification of exon-specific regions from tumour genomic DNA by PCR and for the identification of mutations have been described.[12] PCR was done in a volume of 20 μL with a touchdown PCR programme.[13] Purified PCR products were sequenced with BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analysed with a 3730 ABI capillary electrophoresis system (Applied Biosystems, Foster City). Tumour tissue from patient 13 was limited in quantity, and mutation analyses were therefore not possible for *BRAF* exon 15.

Analyses of *EGFR*

Tissue sections were treated with the procedure used for *ERBB2* with **fluorescence in-situ hybridisation (FISH)** detection kit (DakoCytomation, Glostrup, Denmark). Samples were placed in pretreatment solution for 30 min at 96°C, and digested with pepsin solution for 30 min at room temperature. Dual-colour, dual-target FISH assays were done with the LSI™ EGFR Spectrum Orange/CEP7 Spectrum Green Probe (Vysis, Downers Grove, IL, USA). Tissue sections, covered with 10-μL probe solution, were incubated at 75°C for 5 min to co-denature the *EGFR* and CEP7 (chromosome seven α-centromeric) probes and allowed to hybridise overnight at 37°C. Co-denaturation and hybridisation were done sequentially in a microprocessor-controlled system (Hybridizer, DakoCytomation, Glostrup, Denmark). Posthybridisation stringency wash was done in a water bath at 65°C for 10 min. After washing twice and drying at room temperature for 15 min, tissue sections were covered with 4′6-diamidino-2-phenylindole (DAPI II, Vysis, Downers Glove, IL, USA) for chromatin counterstaining before microscopy.

Analyses were done with a fluorescence microscope (Zeiss Axioskop, Gottingen, Germany) equipped with the Chromowin workstation (Amplimedical, Milan, Italy). *EGFR* was visualised as a red signal with a tetramethylrhodamine isothiocyanate filter, CEP7 as a green signal with a fluorescein isothiocyanate filter, and nuclei as a blue signal with a DAPI filter. Representative images of samples were acquired with a Hamamatsu C5895 chilled CCD camera (Upstate Technical Equipment Co, New York, NY, USA) in monochromatic layers that were subsequently merged by the Casti Imaging FISH Multicolor software (Amplimedical, Milan, Italy).

Two independent observers (SMV and RB) scored at least 200 non-overlapping interphase nuclei for the number of copies of *EGFR* and CEP7 by use of predefined scoring guidelines. The observers were blinded to clinical response of the patients and to each other's assessment and scoring. *EGFR* status was scored as the number of

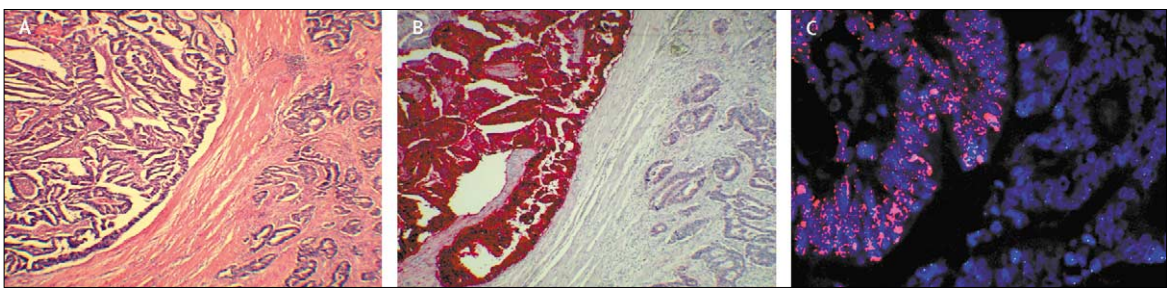


Figure 3: **Non-homogeneous EGFR protein expression corresponding to gene amplification in tumour of patient 10**
Conventional histology (haematoxylin and eosin stain ×100). (B) EGFR overexpression by immunohistochemistry[16] (×100). (C) *EGFR* amplification by FISH in corresponding areas of same tumour (×200). The tumour was EGFR-expressing (B, left side) and EGFR-not-expressing (B, right side).

EGFR signals per nucleus and as the ratio of *EGFR* signals to CEP7 signals. Negative controls consisted of a cultured retinal pigment epithelial (RPE) cell line and healthy colorectal mucosa contiguous to malignant disease for every patient; the control for amplified *EGFR* was the A431 cell line derived from human epidermoid carcinoma. Increased *EGFR* copy number was defined as the presence of three or more signals per nucleus. Samples from patients 4 and 15 were available only as 10-μm sections and despite several attempts, FISH analyses were inconclusive because of excess tissue thickness.

The number of copies corresponding to the *EGFR* locus was determined by real-time quantitative PCR with an ABI PRISM® 7900HT (Applied Biosytems, Foster City). DNA content was normalised to that of Line-1—a repetitive element for which copy numbers per diploid genome are similar in healthy or malignant human cells.[14] Changes in copy number were calculated as: $2^{(Dt-Dline)-(Nt-Nline)}$, where Dt is the mean threshold cycle number for experimental primer in DNA extracted from tumour cells, Dline is the mean threshold cycle number for Line-1 primer in DNA extracted from tumour cells, Nt is the threshold cycle number in reference DNA extracted from RPE cells, and Nline is the threshold cycle number for Line-1 primer in reference DNA extracted from RPE cells.[14] Conditions for amplification were: one cycle of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. PCR for every primer set were done in triplicate, and the mean threshold cycle number was used (panel).

Assay for inhibition of cell proliferation

We assessed the effect of cetuximab on colorectal-cancer cell lines with normal or increased *EGFR* copy number as measured by FISH. Colorectal-cancer cell lines HT-29, HCT-116, DLD-1, SW48, SW480, and LoVo cells were obtained from the ATCC (American Type Culture Collection) repository and DiFi cells (supplied by Jose Baselga, Vall d'Hebron University, Barcelona, Spain) were used to measure inhibition of cell proliferation induced by cetuximab. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics 100 U/mL penicillin, and 100 μg/mL streptomycin), apart from DiFi cells which were grown in F-12 medium supplemented with 10% fetal calf serum and antibitoics. For the assay, cells were grown in DMEM supplemented with 2% fetal bovine serum in 96-well plates (CulturPlate-96F™, Packard Bioscience-PerkinElmer, Milan, Italy), and incubated for 5 days with 0·1–100·0 nmol/L cetuximab (purchased from Komtur Pharmaceuticals, Freiburg, Germany). Incubation was done up to 200·0 nmol/L for SW620 cells because of the high resistance of these cells. Cell proliferation was measured by incorporation of bromodeoxyuridine (BrdU) by use of chemiluminescent ELISA (Roche Applied Science, Monza, Italy). Cell-seeding densities per well were: DiFi, 4000; LoVo, 4000; DLD, 500; HCT116, 1000; HT29, 1000; SW480, 1000; SW387, 4000; SW48, 500; and SW620, 500. The BrdU assay was done in accordance with the manufacturer's instructions and terminated 20 h after addition of labelling solution. Three separate experiments in triplicate were set up for every cell line. The proportion of cell proliferation at various cetuximab concentrations

Fluoresence in-situ hybridisation (FISH)
Hybridisation of cloned fluorescently labelled DNA to intact chromosomes. A general method to assess chromosome location, gene copy number (both increased and decreased), or chromosomal rearrangements.

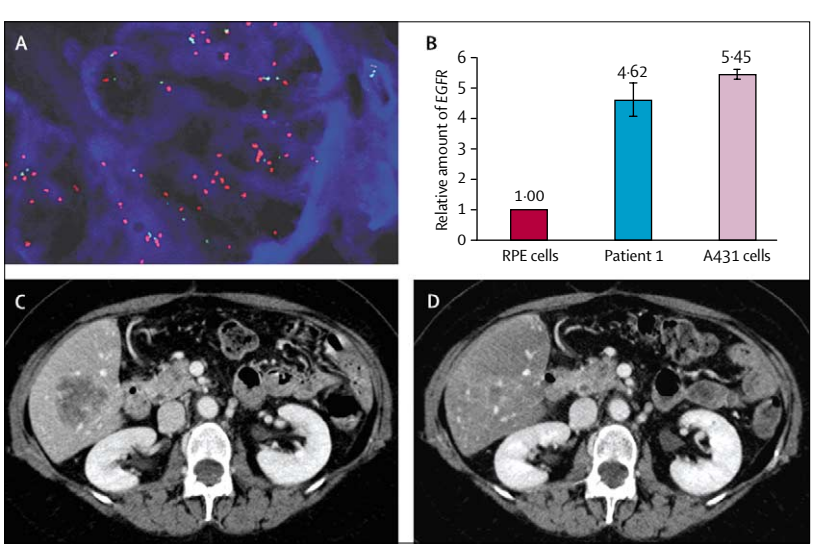
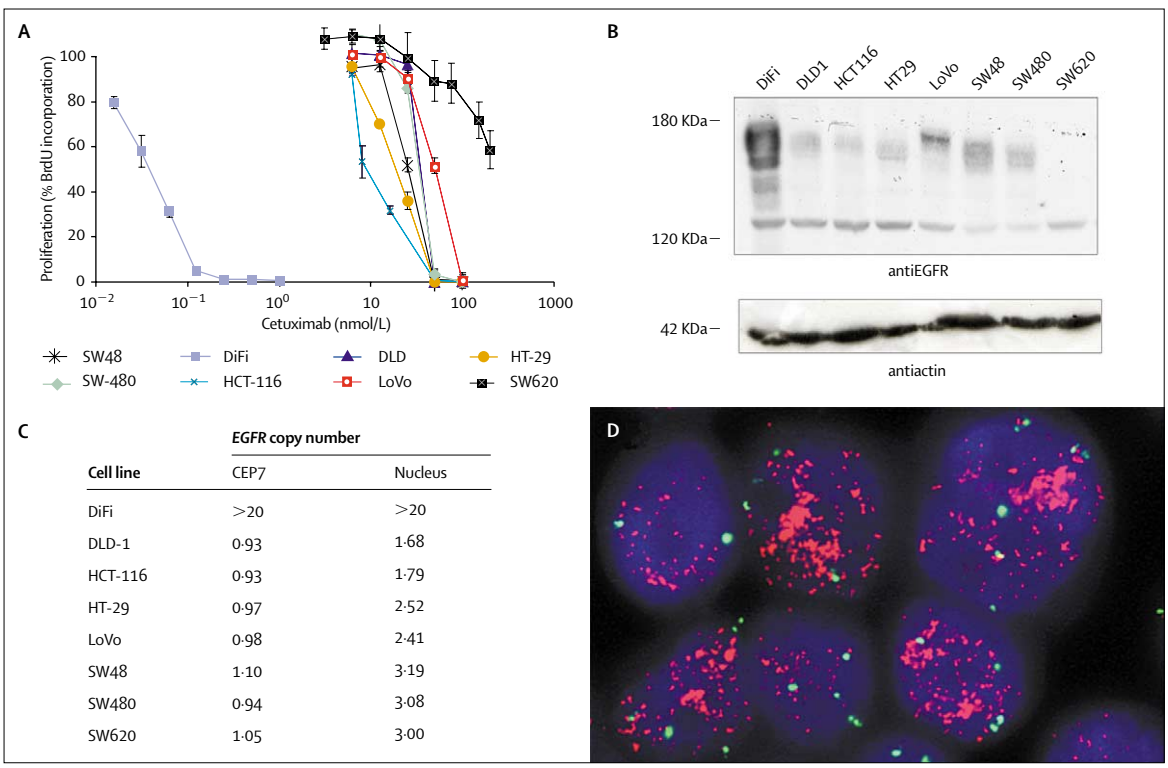


Figure 4: **Molecular changes in *EGFR* and clinical response in patient 1**
(A) Dual-colour FISH assays for *EGFR* (red) and chromosome-7 (CEP7, green) probes showing increased copy number. (B) Relative amount of *EGFR* copies measured by quantitative PCR in tumour of patient 1, A431 cancer-cell line (*EGFR*/nucleus 8·00, *EGFR*/CEP7 2·57), and non-malignant RPE cells (*EGFR*/nucleus 1·60, *EGFR*/CEP7 0·86). Vertical bars are SD. (C) CT showing liver metastasis before (highest diameter, L line 4·4 cm), and (D) after (highest diameter, M line 2·3 cm) treatment with cetuximab.



C

	EGFR copy number	
Cell line	CEP7	Nucleus
DiFi	>20	>20
DLD-1	0·93	1·68
HCT-116	0·93	1·79
HT-29	0·97	2·52
LoVo	0·98	2·41
SW48	1·10	3·19
SW480	0·94	3·08
SW620	1·05	3·00

Figure 5: **Inhibited proliferation of colorectal-cancer cell line by cetuximab**
(A) Mean proliferation of cell lines in three separate experiments (vertical bars are SD) with increasing cetuximab concentration. (B) EGFR protein expression in cell lines measured by western blot. (C) *EGFR* copy number in cell lines as assessed by FISH. (D) Dual-colour FISH assays for *EGFR* (red) and chromosome seven (CEP7, green) probes showing increased copy number in the DiFi cell line.

(test) was calculated by: (test–blank)/(control–blank)×100, where controls were cells grown in medium only (ie, no drug) and blanks were cells grown in 0·02% Triton X in DMEM. Western blotting was done as described.[10]

Statistical analyses

Fisher's exact test was used to calculate p values for *EGFR* copy number by use of STATA 8.2SE. The level of significance was set at p=0·05.

Role of the funding source

The funding sources had no role in study design; collection, analysis, and interpretation of data; or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

The sequencing of *EGFR* exons 18, 19, and 21 showed no somatic mutations, with the exception of patient 13 who had a **missense heterozygous mutation** in exon 21 (Gly857Arg) affecting a residue in the activation loop—a crucial region for catalysis (figure 1). Furthermore, sequencing of the exons of intracellular effectors of *EGFR* identified activating mutations in *KRAS*, *PIK3CA*, and *BRAF* (table 2), but these mutations were not associated with clinical response to monoclonal-antibody (*KRAS* exon 2, p=0·4285; *PIK3CA* exon 9, p=0·3226 and exon 20, p=1·000; *BRAF* exon 15, p=1·000; and all mutations, p=0·2802; tables 1 and 2).

Missense heterozygous mutation
A mutation that changes a codon for one aminoacid into a codon specifying another aminoacid, occurring in one of the two alleles of a gene.

Eight of nine patients with objective responses who were assessable by use of FISH had a ratio of *EGFR* to nucleus that was 3 or more (median ratio 6·80, range 1·65–35·00). By contrast, one of 20 non-responders who were assessable by FISH had increased *EGFR* copy number (median ratio 1·93, range 1·70–20·20). The difference between responders and non-responders was significant (Fisher's exact test, p<0·0001; table 2). Increased *EGFR* copy number was associated with a ratio of *EGFR* to CEP7 of more than two in six of nine assessable patients with a response, thus indicating amplification of *EGFR* by use of criteria used for *ERBB2* assessment.[15] A ratio of *EGFR* to nucleus of 7·10 in patient 3 and of 3·38 in patient 9, and a ratio of *EGFR* to CEP7 of 1·42 in patient 3 and of 1·19 in patient 9 indicate the presence of extra copies of the entire chromosome seven (ie, polysomy seven; figure 2).

The tumour of patient 10 showed a striking amplification of *EGFR* localised into discrete foci, whereas other malignant areas were disomic. Areas of *EGFR* amplification also showed high EGFR expression on immunohistochemical analysis. By contrast, areas

showing disomic *EGFR* amplification did not express the corresponding protein (figure 3).[16]

Quantitative PCR analyses showed an increase in *EGFR* copy number in patient 1, who had responsive disease (figure 4). However, detection of increased copy number in samples from patients with a ratio of *EGFR* to CEP7 of less than three by this method was inconclusive. The proliferation of the DiFi cell line—which had the highest copies of *EGFR*—was substantially inhibited by cetuximab, and the concentration of cetuximab that completely impaired proliferation of DiFi cells did not affect cells with unamplified *EGFR* copy number (figure 5). The SW620 cell line had three copies of *EGFR* and did not express the EGFR protein on western blot (figure 5), a finding which is consistent with a previous report.[17] SW620 cells represent a functional knockout of *EGFR* and thus proliferation is unaffected by cetuximab.

Discussion

We have shown that some patients with metastatic colorectal cancer who have a clinical response to antiEGFR treatment with cetuximab or panitumumab have a significantly increased *EGFR* copy number on assessment of individual tumour samples by FISH. Furthermore, although we detected a new somatic mutation in the *EGFR* catalytic domain and several mutations in immediate downstream effectors (such as *KRAS* and *PIK3CA*), these changes did not correlate with response to monoclonal antibodies against EGFR. These findings are in accordance with a study[18] that found a low frequency of *EGFR* mutations in colorectal cancer, suggesting that such mutations in the EGFR kinase domain are not the basis of the response to monoclonal antibodies in this disease. However, patients in this study were not treated with these agents.

About 10% of patients with metastatic colorectal cancer who are refractory to chemotherapy respond to treatment with panitumumab or cetuximab.[2–4] However, there are no diagnostic tools to identify those likely to benefit from treatment, and thus most patients are exposed to the risk of ineffective therapy with undesired side-effects.[2–4] Non-personalised treatment also results in a substantial financial burden for health-care systems.[19] Our data are consistent with a response of about 10% in patients with non-small-cell lung cancer given the EGFR inhibitors gefitinib or erlotinib. However, responses to these agents in non-small-cell lung cancer have been associated with activating mutations, including in-frame deletions and aminoacid substitutions, in exons 18, 19, and 21 in the catalytic domain of EGFR.[20–22] Of interest, the Gly857Arg mutation is one aminoacid from the Leu858Arg activating mutation noted in gefitinib and erlotinib responders in lung cancer,[20–22] and a mutation affecting the analogous residue in *BRAF* (Gly595Arg) has been identified in colorectal cancers (figure 1).[23]

A key biological implication of our study derives from the observation that most patients with metastatic colorectal cancer who achieve tumour shrinkage with monoclonal antibodies had increased *EGFR* copy number. In these patients, tumour growth is likely to be driven mainly by the EGFR pathway. Together with findings from patients with breast cancer, in which *ERBB2* amplification correlates with sensitivity to trastuzumab,[15] our results suggest that monoclonal antibodies are likely to work most efficiently against targets that are amplified rather than against those affected by point mutations.

Our data also suggest that assessment of *EGFR* copy number by use of FISH might identify patients with metastatic colorectal cancer who are likely to respond to monoclonal antibodies against EGFR. Previous reports of a lack of correlation between clinical response and EGFR expression on immunohistochemical analysis[2–4] might have biological and technical explanations.[1,24] Antibodies used in this type of analysis bind to different epitopes from the ones to which monoclonal antibodies bind, and the therapeutically targeted epitope might be lost during fixation. In the clinical setting, processing and handling of tumour samples is not standardised and if they are not fixed shortly after collection, catalytic degradation of cell-surface receptors might change protein expression, leading to variations in immunohistochemical data. For example, a striking change in EGFR staining intensity with increasing storage time has been shown.[24] Such limitations can be overcome by use of FISH because DNA is a more stable molecule than are proteins.[24] By contrast with semiquantitative assays such as quantitative PCR and western blotting, FISH analysis is not affected by concomitant presence of disomic tumour cells or normal stromal contaminants (as shown by patient 3). Thus, a non-homogeneous pattern of EGFR expression might explain the lack of correlation between immunohistochemical analyses and clinical response to monoclonal antibodies (figure 3 and preliminary data not shown). In view of these findings we are investigating the relation between EGFR expression and gene amplification in the framework of a large prospective clinical trial. Moreover, detection by quantitative PCR of increased gene copy number in samples from patients with a ratio of *EGFR* to CEP7 of less than three was inconclusive, probably because of the limited *EGFR* copy numbers that can be consistently detected with this method.[25,26]

Our findings that cetuximab inhibited proliferation of colorectal-cancer cells with amplified *EGFR* copy number, but that the same dose did not affect cells with unamplified *EGFR* are consistent with previous findings,[17,27] and indicate that colorectal-cancer cells with amplified *EGFR* need this molecular change for proliferation. Our findings lend support to the idea that dependency (or addiction) on such changes in oncogenes are a key feature of human cancers (ie, oncogene addiction) and therefore should be further exploited therapeutically. Furthermore, other genes for tyrosine-kinase receptors closely related to *EGFR*, such as *MET*, have also been found amplified in

patients with metastatic colorectal cancer.[28] Assessment of how these changes stratify with the increase in *EGFR* copy number would be of interest.

In conclusion, we propose a genetically based mechanism to explain the response to antiEGFR treatment and a strategy to identify patients with colorectal cancer who are likely to benefit from monoclonal antibodies. Prospective randomised studies are needed to assess whether selection of patients by use of FISH would affect time to progression or overall survival in patients given monoclonal antibodies. Our findings offer new options for personalised targeted therapy in colorectal cancer on the basis of *EGFR*-amplification status.

Contributors

M Moroni contributed to working hypotheses, clinical care, and writing of the article. S M Veronese did FISH analyses and prepared pathology samples. S Benvenuti did mutational analyses and cellular assays. G Marrapese did data management and tumour-sample collection. A Sartore-Bianchi did data management and clinical care. F Di Nicolantonio did cellular assays and data management. M Gambacorta supervised pathology and FISH analyses. S Siena and Prof A Bardelli are senior authors of the study, and contributed to working hypotheses, obtaining funding support, writing of the article, and supervision of the study. S Siena supervised, and participated in, clinical care.

Conflict of interest

We declare no conflicts of interest.

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