KRAS Mutation Status Is Predictive of Response to Cetuximab Therapy in Colorectal Cancer

Astrid Lièvre,[1,3] Jean-Baptiste Bachet,[3] Delphine Le Corre,[1] Valérie Boige,[4] Bruno Landi,[2] Jean-François Emile,[3] Jean-François Côté,[1,2] Gorana Tomasic,[4] Christophe Penna,[3] Michel Ducreux,[4] Philippe Rougier,[3] Frédérique Penault-Llorca,[5] and Pierre Laurent-Puig[1,2]

[1]Université Paris-Descartes, Institut National de la Sante et de la Recherche Medicale UMR-775; [2]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France; [3]Assistance Publique-Hôpitaux de Paris, Hôpital Ambroise Paré, Boulogne Billancourt, France, Université de Versailles Saint-Quentin-en-Yvelines, Versailles, France; [4]Institut Gustave Roussy, Villejuif, France; and [5]Centre Jean Perrin, Clermont-Ferrand, France, Université Auvergne, Clermont-Ferrand, France

Abstract

The anti-epidermal growth factor receptor (anti-EGFR) cetuximab has been proven to be efficient in metastatic colorectal cancer. The molecular mechanisms underlying the clinical response to this drug remain unknown. Genetic alterations of the intracellular effectors involved in EGFR-related signaling pathways may have an effect on response to this targeted therapy. In this study, tumors from 30 metastatic colorectal cancer patients treated by cetuximab were screened for *KRAS, BRAF*, and *PIK3CA* mutation by direct sequencing and for EGFR copy number by chromogenic *in situ* hybridization. Eleven of the 30 patients (37%) responded to cetuximab. A *KRAS* mutation was found in 13 tumors (43%) and was significantly associated with the absence of response to cetuximab (*KRAS* mutation in 0% of the 11 responder patients versus 68.4% of the 19 nonresponder patients; $P = 0.0003$). The overall survival of patients without *KRAS* mutation in their tumor was significantly higher compared with those patients with a mutated tumor ($P = 0.016$; median, 16.3 versus 6.9 months). An increased *EGFR* copy number was found in 3 patients (10%) and was significantly associated with an objective tumor response to cetuximab ($P = 0.04$). In conclusion, in this study, *KRAS* mutations are a predictor of resistance to cetuximab therapy and are associated with a worse prognosis. The *EGFR* amplification, which is not as frequent as initially reported, is also associated with response to this treatment. (Cancer Res 2006; 66(8): 3992-5)

Introduction

Colorectal cancer is one of the most common human malignancies with >300,000 cases both in the United States and in the European Union each year. In the past decade, survival of metastatic colorectal cancer patients has approximately doubled. This significant improvement is mainly due to the development of new combinations of standard chemotherapy, including 5-fluorouracil, irinotecan, and oxaliplatin, and also to the introduction of new targeted therapies, such as monoclonal antibodies against epidermal growth factor receptor (EGFR) or monoclonal antibodies against vascular endothelial growth factor. The addition of such targeted therapies to standard chemotherapy regimens results in an increase of toxicity and treatment costs (1) and therefore requires the identification of decision-making tools to select patients who are likely to benefit from them. The chimeric IgG1 monoclonal antibody cetuximab has been proven efficient in irinotecan-resistant metastatic colorectal cancer expressing the EGFR by immunohistochemistry, with response rates ranging between 8.8% when used in monotherapy and 22.9% when combined with irinotecan (2, 3). However, the molecular mechanisms underlying the clinical response or resistance to this drug remain unknown. Recently, a 25% objective response rate was obtained in a series of colorectal cancers that did not express EGFR by immunohistochemistry (4), highlighting the potential existence of other predictive markers of response to cetuximab.

Recent progresses have been made in the understanding of the EGFR pathway involved in colorectal carcinogenesis. The binding of a ligand on the extracellular part of EGFR results in the phosphorylation of the tyrosine kinase domain located in its intracellular part. Then, the activation of the receptor leads to the activation of intracellular effectors involved in intracellular signaling pathways, such as the G protein K-ras, the protein kinase RAF [Ras/mitogen-activated protein kinase (MAPK) pathway], and phosphoinositide 3-kinase (PI3K/Akt pathway). Cetuximab binds to EGFR with a high specificity and blocks ligand-induced phosphorylation of the receptor. Consequently, we hypothesized that mutation in the *KRAS*, *BRAF*, and *PI3KCA* coding genes could affect the clinical response to this monoclonal antibody. An analysis of the *EGFR* copy number was simultaneously done as a correlation between *EGFR* amplification and response to anti-EGFR therapy was recently reported (5).

Patients and Methods

Patients. We assessed 30 metastatic colorectal cancer patients (19 males and 11 females; mean age, 62.3 ± 10.9 years) treated by antibodies against EGFR cetuximab (Erbitux, Merck, Lyon, France) in three centers (Hôpital Ambroise Paré, Boulogne Billancourt; Hôpital Européen Georges Pompidou, Paris; Institut Gustave Roussy, Villejuif). All these patients had a metastatic colorectal adenocarcinoma histologically proved and underwent a surgical resection of their primary tumor. An analysis of EGFR expression was done by immunohistochemistry on each primary tumor that was considered EGFR positive if at least 1% malignant cells stained for EGFR (Zymed Laboratories, Inc., San Francisco, CA or DakoCytomation, Glostrup, Denmark). Patients' inclusion in this study was based on the availability of sufficient frozen tumor tissues and the existence of a signed informed consent.

One patient received cetuximab monotherapy, 25 received cetuximab combined with irinotecan alone, and four received cetuximab combined with irinotecan plus 5-fluorouracil and folinic acid (FOLFIRI regimen;

Note: A. Lièvre and J-B. Bachet equally contributed to the work.

Requests for reprints: Pierre Laurent-Puig, Institut National de la Sante et de la Recherche Medicale U775, Molecular Basis of Response to Xenobiotics, 45 rue des Saints-Pères, 75006 Paris, France. Phone: 33-1-4286-2081; Fax: 33-1-4286-2072; E-mail: pierre.laurent-puig@univ-paris5.fr.

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Table 1). Cetuximab was given as first-line treatment in three cases (EMR 62202-010 phase II trial), as second-line in three cases, and as third-line or more in 24 cases after disease progression under irinotecan-based chemotherapy.

Tumor response was evaluated by computerized tomodensitometry according to the Response Evaluation Criteria in Solid Tumors criteria and classified in complete response, partial response, stable disease, and progressive disease. For the analysis, complete and partial responder patients were grouped in responder; patients with a stable and a progressive disease were grouped in nonresponder patients.

DNA extraction and mutation analysis. DNA was extracted from frozen colorectal cancer samples using QIAmp DNA Mini kit (Qiagen,

Table 1. Clinical characteristics, tumor mutation analysis, and *EGFR* copy number analysis in metastatic colorectal cancer patients treated by cetuximab

Sex	Age (y)	Previous chemotherapy regimens for metastastic disease	Anti-EGFR treatment	Tumor response		*KRAS* mutation	*PIK3CA* mutation	*EGFR* copy number*
				Best response	Duration (wk)			
M	77	FOLFIRI	Cetuximab and irinotecan	CR	58.1	WT	WT	20
M	61	NA	Cetuximab and FOLFIRI	PR	34.1	WT	WT	11
M	76	NA	Cetuximab and FOLFIRI	PR	33.9	WT	WT	2.6
M	67	NA	Cetuximab and FOLFIRI	PR	20.9	WT	WT	3.7
M	71	LV5FU2, FOLFOX, capecitabine and mitomycin, capecitabine and irinotecan, irinotecan	Cetuximab and irinotecan	PR	46.0	WT	WT	3
F	44	LV5FU2 IV and oxaliplatin IAH, FOLFOX IV and Adriamycin IAH, FOLFIRI, irinotecan	Cetuximab and irinotecan	PR	62.9	WT	WT	3
M	72	LV5FU2, LV5FU2 IV and oxaliplatin IAH, FOLFIRI, capecitabine and irinotecan	Cetuximab and irinotecan	PR	44.0	WT	WT	2.1
M	48	LV5FU2, FOLFOX, raltitrexed and oxaliplatin, FOLFIRI, phase I trial, capecitabin and mitomycin	Cetuximab and irinotecan	PR	17.1	WT	WT	11
F	55	FOLFIRI, FOLFOX, irinotecan,	cetuximab	PR	23.7	WT	WT	3.4
F	64	FOLFOX, FOLFIRI	Cetuximab and irinotecan	PR	17.1	WT	WT	2.5
M	62	LV5FU2 IV and oxaliplatin IAH, FOLFOX, FOLFIRI	Cetuximab and irinotecan	PR	32.0	WT	WT	2.8
M	50	FOLFIRI, FOLFIRI IV and oxaliplatin IAH, irinotecan	Cetuximab and irinotecan	SD	14.7	WT	WT	2.9
M	54	FOLFOX, FOLFIRI	Cetuximab and FOLFIRI	SD	20.0	G12S	WT	2.6
F	73	LV5FU2, FOLFIRI, FOLFOX	Cetuximab and irinotecan	SD	19.3	G12D	E542K	2.75
M	71	raltitrexed and oxaliplatin, raltitrexed and irinotecan	Cetuximab and irinotecan	SD	16.0	G13D	WT	2.8
F	53	FOLFOX, FOLFIRI, irinotecan	Cetuximab and irinotecan	SD	20.0	G12A	WT	2.25
F	73	FOLFOX	Cetuximab and irinotecan	SD	20.0	WT	WT	2.25
M	78	FOLFOX, FOLFIRI	Cetuximab and irinotecan	PD		WT	WT	2.4
F	51	FOLFIRI, FOLFOX	Cetuximab and irinotecan	PD		G12D	WT	3.2
F	75	FOLFIRI, oxaliplatin and capecitabine	Cetuximab and irinotecan	PD		G12V	E542K	2.3
M	69	LV5FU2 and oxaliplatin and irinotecan, FOLFIRI	Cetuximab and irinotecan	PD		G13D	WT	3.3
M	72	LV5FU2, FOLFOX, FOLFIRI IV and oxaliplatin IAH	Cetuximab and irinotecan	PD		WT	WT	3.8
F	61	LV5FU2 and oxaliplatin and irinotecan, irinotecan	Cetuximab and irinotecan	PD		WT	WT	2.3
M	53	FOLFOX, FOLFIRI, raltitrexed and oxaliplatin, capecitabine, irinotecan	Cetuximab and irinotecan	PD		G12D	WT	2.3
M	59	FOLFIRI, FOLFOX	Cetuximab and irinotecan	PD		G12D	WT	2.9
M	75	FOLFOX, FOLFIRI	Cetuximab and irinotecan	PD		G12D	WT	2.7
F	58	FOLFOX, FOLFIRI, LV5FU2 and mitomycin, FOLFOX, phase I trial	Cetuximab and irinotecan	PD		G12C	WT	3.7
M	47	LV5FU2, FOLFOX, FOLFIRI	Cetuximab and irinotecan	PD		WT	WT	3.3
M	41	capecitabine and oxaliplatin	Cetuximab and irinotecan	PD		G12C	WT	3.6
F	60	FOLFIRI, FOLFOX, irinotecan	Cetuximab and irinotecan	PD		G13D	WT	2.7

Abbreviations: M, male; F, female; IAH, intra-artery hepatic infusion; IV, i.v. infusion; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NA, not applicable; FOLFOX, oxaliplatin, fluorouracil, and folinic acid; FOLFIRI, irinotecan, fluorouracil, and folinic acid; WT, wild type.

*Gene copy number per nucleus.

Courtaboeuf, France) after a histologic control of the presence of tumor cells (>70%) in each tumor fragment by HES coloration.

Exon 1 of the *KRAS* gene, exons 11 and 15 of the *BRAF* gene, and exons 1, 2, 9, and 20 of the *PIK3CA* gene were selected for mutation analysis because they were frequently found mutated in colorectal cancer (6). These exons were sequenced after PCR amplification. Primers used for the amplification and sequencing of exon-specific region of each gene and the PCR conditions are available upon request. Direct sequencing was done using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI Prism 3900 DNA Analyzer automated sequencer (Applied Biosystems). All somatic mutations found were further validated by a new independent amplification and sequencing.

Analysis of EGFR amplification by chromogenic *in situ* hybridization. Chromogenic *in situ* hybridization (CISH) EGFR experiments were done according to the protocol given by the manufacturer (Invitrogen-Zymed, Carlsbad, CA) on formalin-fixed, paraffin-embedded tumor specimens. Codenaturation of EGFR SPOT-Light probe and DNA target and hybridization were made on a HYBrite instrument (Vysis-Abbott Diagnostic, Baar, Switzerland). CISH results were evaluated with a light microscope using a ×40 dry objective or a ×60 oil objective.

Statistical analysis. Fischer's exact test was used to calculate p value for association between *KRAS, BRAF*, and *PIK3CA* mutation and response to cetuximab. A logistic regression was done to estimate the hazard ratio of response according to the *KRAS* mutation status. The survival rates were calculated with the Kaplan-Meier method. Survival curves were compared using the log-rank test. Analysis was carried out using the STATA software package (College Station, TX). The level of significance was set at $P = 0.05$. *P*s were not corrected for multiple comparisons.

Results and Discussion

Eleven of the 30 patients (37%) responded to cetuximab (Table 1). The median duration of response to cetuximab was 33.9 months (range, 17.1-62.9 months). In the six patients with stable disease under cetuximab treatment, the median duration of stabilization was 18.3 months (range, 14.7-20.0 months).

A *KRAS* mutation was found in the tumor of 13 patients (43%; Fig. 1*A* and *B*). No tumor had a *BRAF* mutation, which is consistent with the absence of microsatellite instability determined by the genotyping of five microsatellites in all the tumors included in our series. A *PIK3CA* mutation, located in the exon 9, was found in two tumors (7%), which also had a *KRAS* mutation.

No *KRAS* mutation was found in the tumor from 11 patients with a clinical response to cetuximab [0%; 95% confidence interval (95% CI), 0-28.5%], whereas 13 tumors from the 19 nonresponder patients (68.4%; 95% CI, 43.5-87.5%) were found mutated in this gene ($P = 0.0003$). Therefore, the presence of *KRAS* mutation was significantly associated with the absence of response to cetuximab. This difference remained significant even if the three responder patients treated with the association of cetuximab and FOLFIRI regimen as first-line treatment were removed from the analysis (*KRAS* mutation: 0 of 8 responders versus 13 of 19 nonresponders; $P = 0.002$). In this group of 27 patients, the overall survival of patients without *KRAS* mutation in their tumor was significantly higher compared with those patients with a mutated tumor ($P = 0.016$; median, 16.3 versus 6.9 months; Fig. 2). No significant correlation was found between *PIK3CA* mutation and response to cetuximab. In our series, an increased *EGFR* copy number was found by CISH in 3 of 30 (10%) patient tumors (Table 1; Fig. 1*C*). Amplification was defined as six or more signals per nucleus in >50% of cancer cells, or when a large gene copy cluster was seen. At least 30 nuclei were counted per slide. Slides were scored in a blinded manner by two pathologists. All the patients with *EGFR* amplification were responders, leading to a significant association between *EGFR* amplification and response to cetuximab (*EGFR* amplification in responder versus nonresponder patients: 27% versus 0%; $P = 0.04$).

One study had previously assessed the mutation status of the *EGFR* catalytic domain and its downstream intracellular effectors *PIK3CA, KRAS*, and *BRAF* and found no significant correlation with response to cetuximab (5). However, a potential trend toward higher response rates was seen in cetuximab-treated colorectal cancer patients whose tumors were of *KRAS* wild-type status. When our results were pooled with those of Moroni et al., the predictive value of *KRAS* mutation remained significant with a *KRAS* mutation frequency of 52.5% in nonresponders compared with 9.5% in responders ($P = 0.001$). When considering these pooled data, the probability to have no response to cetuximab was 91.3% in the presence of *KRAS* mutation. Moreover, the probability to be responder was 50% when no *KRAS* mutation was identified. The relative risk to obtain a response to cetuximab was 10-fold higher for nonmutated patients compared with that of patients with *KRAS* mutation (hazard ratio, 10.5; 95% CI, 2.1-51.1). Thus, these data suggest that the wild-type *KRAS* status might identify patients with metastatic colorectal cancer who are likely to respond to cetuximab and to have a longer overall survival.

Moroni et al. showed that the gene copy number for *EGFR* determined by fluorescence *in situ* hybridization (FISH) on tumor samples was significantly associated with clinical response to this targeted therapy (5). However, the prevalence of *EGFR* amplification in their series (31%) was much higher than the prevalence we

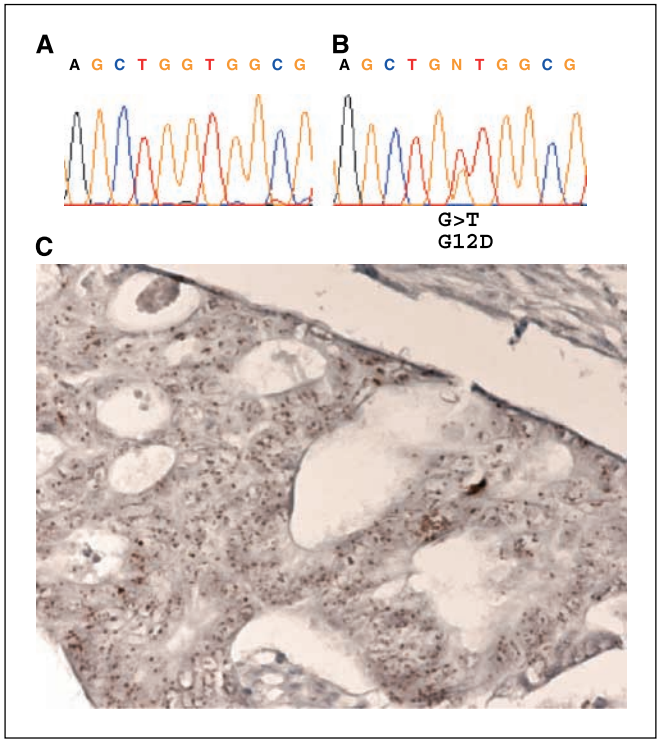

Figure 1. Example of different genetic alterations studied. *A* and *B*, electrophoregram from normal (*A*) and tumor tissue (*B*). A G12D *KRAS* mutation is observed in tumor tissue compared with normal tissue. *C*, an example of high *EGFR* amplification by chromogenic in situ hybridization. Original magnification, ×100. One brown spot corresponds to one *EGFR* gene copy.

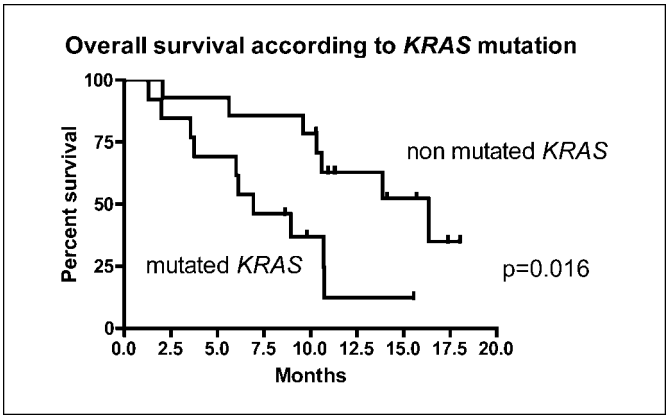

Figure 2. Overall survival curves of patients with a *KRAS*-mutated and nonmutated tumor.

observed. Our results are in accordance with a recent report by Shia et al. (7), showing a copy gain in only 11.5% of 147 colorectal cancers tested by CISH. Furthermore, Sauer et al. (8) reported a prevalence of 15% in a series of 48 rectal cancers tested by FISH. These discrepancies question the prevalence of increased *EGFR* copy number in colorectal cancer and its relevance for the prediction of response to cetuximab. However, it is interesting to note that the unique complete responder patient in our series is the one who have the higher level of *EGFR* amplification. This is in good agreement with the *in vitro* proliferation inhibition observed with a low dose of cetuximab in the DiFi tumor cell line that has the highest copy number of *EGFR* of the different tested cell lines (5).

Our results suggest that cetuximab should not be proposed to ~40% of all metastatic colorectal cancer patients having *KRAS*-mutated tumor cells. Previous studies have shown a lack of correlation between EGFR expression on immunohistochemical analysis and response to cetuximab with a 22.9% maximal response rate in EGFR-expressing colorectal cancer (2). The presence of a *KRAS* mutation in EGFR-positive colorectal cancers might partially explain why one part of these tumors does not respond to cetuximab. K-ras is a G protein that plays a key role in the Ras/MAPK signaling pathway located downstream of many growth factor receptors, including EGFR, and involved in colorectal carcinogenesis. The recruitment of K-ras by the activated EGFR is responsible for the activation of a cascade of serine-threonine kinases from the cell surface to the nucleus. The Ras/MAPK pathway is one of the most important pathways for cell proliferation by inducing the synthesis of cyclin D1 and mutation of the *KRAS* proto-oncogene, which are found in 36% of colorectal cancers (9), leading to the activation of this pathway. Consequently, we can hypothesize that whatever the expression level of EGFR is, the presence of a *KRAS* mutation is associated with a downstream activation of the Ras/MAPK pathway, leading to cell proliferation that cannot be significantly inhibited by cetuximab that acts upstream of the K-ras protein. In accordance with our results, *KRAS* mutations were found to be associated with resistance to EGFR kinase inhibitors gefitinib and erlotinib in lung adenocarcinomas (10). A similar trend was recently observed in a series of 30 colorectal cancers treated with gefitinib, among which the response rate for tumors with and without *KRAS* mutation was 33% and 47%, respectively (11).

In conclusion, we have shown that *KRAS* mutation is associated with resistance to cetuximab and a shorter survival in EGFR-positive metastatic colorectal cancer patients treated with this therapy. Thus, *KRAS* mutation status might allow the identification of patients who are likely to benefit from cetuximab and avoid a costly and potentially toxic administration of this treatment in nonresponder patients. Prospective randomized study is needed to validate this result that bring a new possibility of targeted therapy adapted to each patient according to its *KRAS* mutation status.

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