Primary and Acquired Resistance of Colorectal Cancer to Anti-EGFR Monoclonal Antibody Can Be Overcome by Combined Treatment of Regorafenib with Cetuximab

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Abstract

Purpose: In colorectal cancer, the activation of the intracellular RAS–RAF and PIK3CA–AKT pathways has been implicated in the resistance to anti-EGFR mAbs. We have investigated the role of regorafenib, an oral multikinase inhibitor, in combination with cetuximab, an anti-EGFR mAb, to overcome anti-EGFR resistance.

Experimental Design: We have tested, in vitro and in vivo, the effects of regorafenib in a panel of human colorectal cancer cell lines with a KRAS mutation (SW480, SW620, HCT116, LOVO, and HCT15) or with a BRAF mutation (HT29), as models of intrinsic resistance to cetuximab treatment, and in two human colorectal cancer cell lines (GEO and SW48) that are cetuximab-sensitive, as well as in their derived cells with acquired resistance to cetuximab (GEO-CR and SW48-CR).

Results: Treatment with regorafenib determined a dose-dependent growth inhibition in all colorectal cancer cell lines. The combined treatment with cetuximab and regorafenib induced synergistic antiproliferative and apoptotic effects in cetuximab-resistant cell lines by blocking MAPK and AKT pathways. Nude mice were injected s.c. with HCT116, HCT15, GEO-CR, and SW48-CR cells. The combined treatment caused significant tumor growth inhibition. Synergistic antitumor activity of regorafenib plus cetuximab was also observed in an orthotopic colorectal cancer model of HCT116 cells. In particular, the combined treatment induced a significant tumor growth inhibition in the primary tumor site (cecum) and completely prevented metastasis formation.

Conclusions: The combined treatment with cetuximab and regorafenib could be a strategy to overcome resistance to anti-EGFR therapies in metastatic colorectal cancer patients. Clin Cancer Res; 21(13); 2975–83. ©2015 AACR.

Introduction

Colorectal cancer is one of the leading causes of cancer-related mortality worldwide, with more than 1.2 million new cases and 608,700 deaths estimated in 2008 (1). Despite improvements made in screening strategies, a significant number of patients are still diagnosed at late stages of the disease.

In the last decade, the introduction of targeted therapies in clinical practice, in particular of agents targeting the VEGF-related pathway (bevacizumab and aflibercept) and the EGFR (cetuximab and panitumumab) has changed the therapeutic approach to metastatic colorectal cancer patients, with a significant improvement in progression-free survival (PFS) and overall survival (OS; ref. 2). Cetuximab and panitumumab are mAbs that block the activation of the EGFR and of its downstream intracellular signals, the RAS–RAF–MEK–MAPK and the PTEN–PIK3CA–AKT pathways (3–6). These two drugs are currently approved for the treatment of metastatic colorectal cancer patients with all-RAS wild-type tumors. Nevertheless, prognosis remains poor for most of these patients. In fact, the use of these mAbs is limited by the presence of preexisting intrinsic resistance mechanisms or by the ability of cancer cells to acquire resistance. Possible mechanisms for primary and acquired resistance to cetuximab include mutations in the KRAS, BRAF, and NRAS genes, secondary mutation (S492R) in the extracellular domain of EGFR, HER2 gene amplification, and/or increased HER2 signaling and overexpression of the MET pathway (7–10).

Recently, it has been elucidated that in the resistance to anti-EGFR therapies, different growth factors and receptors could be activated in the cancer cell to drive alternative signaling pathways that bypass the EGFR (11, 12). Molecular heterogeneity also plays an important role in the context of resistance, by limiting the success of therapies against a single target. Colorectal cancer patients can harbor different gene mutations in
distinct tumor lesions, or even within different regions of the same lesion (13). All these alterations could converge on activation of the RAS–MEK–ERK pathway (9, 10, 14, 15). Understanding the biology of such complex gene heterogeneity in tumors is necessary for developing rational combination therapies. In fact, blockade of multiple growth factor and growth factor receptor pathways could be needed to increase the efficacy of anti-EGFR mAbs. In this study, we have demonstrated that, in human colorectal cancer cells with either primary or acquired resistance to cetuximab, the combined treatment with cetuximab and regorafenib induces synergistic antiproliferative and apoptotic effects and causes significant tumor growth inhibition. This study provides a rationale for evaluating combined treatment with cetuximab and regorafenib as a therapeutic strategy for preventing and/or overcoming cetuximab resistance in metastatic colorectal cancer patients.

Translational Relevance

The introduction in clinical practice of mAbs against the EGFR, such as cetuximab or panitumumab, in combination with chemotherapy has demonstrated therapeutic efficacy in metastatic colorectal cancer patients with all RAS wild-type tumors. However, efficacy of these mAbs is limited by development of resistance mechanisms in cancer cells. Activation of alternative signaling pathways that bypass the EGFR has been implicated in the resistance to anti-EGFR therapies. Therefore, the blockade of multiple growth factor and receptor pathways could be necessary to increase the efficacy of anti-EGFR mAbs. In this study, we have demonstrated that, in human colorectal cancer cells with either primary or acquired resistance to cetuximab, the combined treatment with cetuximab and regorafenib induces synergistic antiproliferative and apoptotic effects and causes significant tumor growth inhibition. This study provides a rationale for evaluating combined treatment with cetuximab and regorafenib as a therapeutic strategy for preventing and/or overcoming cetuximab resistance in metastatic colorectal cancer patients.

Materials and Methods

Drugs

Cetuximab, an anti-EGFR human-mouse chimeric mAb, was kindly provided by Merck Serono Italy, and it was ready to use. Regorafenib was kindly provided by Bayer Pharma Italy. For in vitro applications, regorafenib was dissolved in sterile DMSO, and the 10 mmol/L stock solution was stored in aliquots at −20°C. Working concentrations were diluted in culture medium just before each experiment. For in vivo applications, regorafenib was solubilized in 0.5% Tween-80 in sterile PBS.

Cell lines

The human HT29, SW620, LOVO, and HCT15 colorectal cancer cell lines were obtained from the ATCC and have been authenticated by IRCCS “Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca del Cancro, Genova” Italy. The human SW48 (catalogue number: HTL99020), SW480 (catalogue number: HTL95025), and HCT116 (catalogue number: HTL99017) colorectal cancer cell lines were obtained from IRCCS “Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca del Cancro, Genova” Italy. The human GEO colon cancer cell line was kindly provided by Dr. N. Normanno (National Cancer Institute, Naples, Italy). GEO-CR and SW48-CR cells were established as previously described (10, 14, 21). GEO and GEO-CR cell lines were grown in DMEM (Lonza), supplemented with 10% FBS (Lonza), 1% penicillin–streptomycin (Lonza). SW48, SW480, HCT116, LOVO, HCT15, and SW48-CR cell lines were grown in RPMI-1640 (Lonza) supplemented with 10% FBS, 1% penicillin–streptomycin. SW620 and HT29 cancer cells were grown in McCoy medium (Lonza) supplemented with 20% FBS (Lonza), 1% penicillin–streptomycin (Lonza). All cell lines were grown in a humidified incubator with 5% of carbon dioxide (CO₂) and 95% air at 37°C. All cell lines were routinely screened for the presence of Mycoplasma (“Mycoplasma Detection Kit; Roche Diagnostics”)

Proliferation assay

Cancer cell lines were seeded in 24-well plates and were treated with different concentrations of cetuximab (range, 0.001–20 μg/mL) alone or in combination with regorafenib (range, 0.001–5 μmol/L) for 96 hours. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The IC₅₀ value was determined by interpolation from the dose–response curves. Results represent the median of three separate experiments, each performed in quadruplicate. Results of the combination treatment were analyzed according to the method of Chou and Talalay by using the CalcuSyn software program (Biosoft).

Apoptosis assay

HT29, SW480, SW620, HCT116, LOVO, HCT15, GEO-CR, and SW48-CR cells were seeded in 6-well plates, treated with cetuximab, regorafenib or their combination at different concentrations 72 hours and stained with Annexin V–FITC (Invitrogen). Apoptotic cell death was assessed by counting the numbers of cells that stained positive for Annexin V–FITC using an Apoptosis Annexin V–FITC Kit (Invitrogen), coupled with FACS analysis, by following the manufacturer’s protocol.
Immunoblotting

SW480, SW620, HCT116, LOVO, HCT15, GEO-CR, and SW48-620 cells were seeded into 100 mm dishes and treated with vehicle, cetuximab, regorafenib, or their combination for 24 hours at different concentration as following indicated. Fifty mg of protein lysates, estimated by a modified Bradford assay (Bio-Rad), were subjected to Western blot analysis.

Orthotopic colorectal cancer model

To evaluate the statistical significance of the results, we performed the following:

1. Sensitivity to cetuximab and regorafenib treatment in a panel of human colorectal cancer cell lines
2. Orthotopic colorectal cancer model

**Results**

**Sensitivity to cetuximab and regorafenib treatment in a panel of human colorectal cancer cell lines**

We first tested in vitro the activity of cetuximab and regorafenib, as single agents, in a panel of human colorectal cancer cell lines to characterize their spectrum of activity. We selected eight human colorectal cancer (GEO, SW48, HT29, SW480, SW620, HCT116, LOVO, and HCT15) cell lines, having different mutation profiles.

**Orthotopic colorectal cancer model**

Four- to 6-week-old female balb/c athymic (nu+/nu−) mice were purchased from Charles River Laboratories. The research protocol was approved and mice were maintained in accordance with institutional guidelines. All procedures involving laboratory animals were in accordance with institutional guidelines and with the approval of the Institutional Animal Care and Use Committee. Male mice were acclimatized at the facility for 1 week before being injected with cancer cells and then caged in groups of five under controlled conditions (12–12 hours light-dark cycle; room temperature 20 ± 2°C; humidity 55%–60%). A total number of 3.5 × 10⁶ GEO-CR, SW48-CR cells, and 2 × 10⁶ HCT116, HCT15 cells in 200 μL of Matrigel (BD Biosciences)/PBS (1:1) were s.c. injected to the dorsal flank of mice. The mean values of tumors were between 200 and 300 mm², mice were randomly assigned to one of the following groups (10 mice/group). Group 1, vehicle administrated orally and i.p. Group 2, cetuximab injected twice a week i.p. at the dose of 1 mg for 3 weeks. Group 3, regorafenib administrated by oral daily gavage at the dose of 10 mg/kg for 3 weeks. Group 4, combination of regorafenib and cetuximab. Monitoring of tumor growth was performed until tumors reached approximately 2,000 mm³ when mice were euthanized. Tumor size was evaluated twice a week by caliper measurements using the following formula: π/6 × larger diameter × (smaller diameter)². The Student t test was used to evaluate the statistical significance of the results.
obtained in our laboratory (10, 14, 21). As shown in Fig. 1, regorafenib shows a different proliferation inhibitory effect in these human colorectal cancer cell lines, with IC50 values ranging between 0.5 μmol/L (HCT116, HT29, and LOVO), 1 μmol/L (GEO), 2 μmol/L (SW480 and HCT15), and >2 μmol/L (SW48, SW620, SW48-CR, and GEO-CR). No significantly differences in regorafenib efficacy were observed among colorectal cancer cell lines harboring KRAS, NRAS, BRAF, PIK3CA mutations, indicating that its antitumor activity seems to be independent of the molecular profile of colorectal cancer cell lines tested.

**Effects of cetuximab in combination with regorafenib on intracellular signaling pathways in a panel of human colorectal cancer cell lines**

We evaluated the antiproliferative activity of cetuximab and regorafenib in combination in the panel of human colorectal cancer cell lines (Supplementary Figs. S1 and S2). Combination index (CI) values were calculated according to the Chou and Talalay mathematical model for drug interactions using the CalcuSyn software, as previously described (10, 14, 24, 27). A synergistic growth inhibitory effect was observed in human colorectal cancer cell lines with both primary and acquired resistance to cetuximab. In fact, the CI values for the combined treatments were significantly <1.0 for all the drug doses tested (CI values ranging between 0.0001 and 0.7; Supplementary Figs. S1 and S2). In contrast, an antagonistic effect of the combined treatment was observed in sensitive colorectal cancer cell lines (GEO and SW48) with CI values significantly >1.0 (data not shown).

**Effects of cetuximab in combination with regorafenib on intracellular signaling pathways in a panel of human colorectal cancer cell lines**

To examine the mechanism by which the combined treatment contributes to inhibition of proliferation in colorectal cancer cell lines with primary or acquired resistance to anti-EGFR inhibitor, the activation of EGFR downstream signaling molecules was evaluated. SW480, SW620, HCT116, LOVO, HCT15, SW48-CR, and GEO-CR cells were treated with cetuximab, regorafenib and/or their combination. The activation of PIK3CA–AKT and RAS–MAPK pathways was analyzed by Western blotting. The combined treatment with cetuximab and regorafenib substantially inhibited
Regorafenib and Cetuximab Overcome Resistance to Cetuximab

Figure 2.
Effects of cetuximab in combination with regorafenib on intracellular signaling pathways in a panel of colorectal cancer cell lines with primary and acquired resistance to anti-EGFR inhibitor. Cells were treated with cetuximab at a dose of 1 mg/mL, with regorafenib at a dose of 1 μmol/L, or with their combination for 24 hours. Total cell protein extracts (50 μg) were subjected to immunoblotting with the indicated Abs, as described in Materials and Methods. Antitubulin antibody was used for normalization of protein extract content. Experiments were repeated three times.

Proapoptotic effect of cetuximab in combination with regorafenib in colorectal cancer cell lines with primary and acquired resistance to anti-EGFR drugs

We measured the ability of cetuximab and regorafenib as single agents or in combination, to induce apoptosis in colorectal cancer cell lines by the Annexin V–FITC assay (Table 1 and Supplementary Fig. S3). Compared with single agent, the combined treatment induced significantly early and late apoptosis in the whole cell population. Cell death was determined by flow cytometry as Annexin V–FITC positive cells.

Table 1. Proapoptotic effects of cetuximab in combination with regorafenib in colorectal cancer cell lines with primary and acquired resistance to anti-EGFR inhibitor

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Apoptotic cells (24 h), %</th>
<th>Cell line</th>
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<tbody>
<tr>
<td>HT29</td>
<td>CTR</td>
<td>14%</td>
<td>LOVO</td>
<td>CTR</td>
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<td>Combination</td>
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<td>SW480</td>
<td>CTR</td>
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<td>Combination</td>
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<tr>
<td>SW620</td>
<td>CTR</td>
<td>14%</td>
<td>SW48-CR</td>
<td>CTR</td>
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<td>HCT116</td>
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<td>10%</td>
<td>GEO-CR</td>
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NOTE: The rate of apoptosis was expressed as a percentage of the total cells counted.

phosphorylation of both AKT and MAPK after 24 hours of treatment compared with single-agent treatments (Fig. 2). A strong reduction of phosphorylated S6 ribosomal protein (pS6) levels, the major downstream effect of AKT/m-TOR signaling, was observed in the combination treatment (Fig. 2). These findings suggested that cetuximab in combination with regorafenib could overcome resistance to anti-EGFR treatment by inhibiting PIK3CA–AKT and MAPK pathways.

Table 1. Proapoptotic effects of cetuximab in combination with regorafenib in colorectal cancer cell lines with primary and acquired resistance to anti-EGFR inhibitor

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panel of human colorectal cancer cell lines with primary or acquired resistance to cetuximab.

Cetuximab plus regorafenib combination exhibits antitumor activity in subcutaneous colorectal cancer xenograft models

We evaluated the in vivo activity of cetuximab alone or in combination with regorafenib in nude mice s.c. injected with cetuximab-resistant HCT15, HCT116, GEO-CR, or SW48-CR cell lines. Mice were randomly assigned to receive vehicle, cetuximab, regorafenib, or their combination and were treated for 3 weeks. As shown in Fig. 3, treatment with cetuximab had little or no effect on tumor growth in all tumor xenografts. Similar results were obtained in the groups treated with regorafenib alone. On the contrary, the combined treatment significantly inhibited tumor growth compared with both the control group and to single-agent treatments in all tumor xenografts (Fig. 3). Single-agent and combination treatment protocols were well tolerated by mice and were not accompanied by any major side effect or treatment-related weight loss. No cellular abnormalities were observed in the examined organs, including heart, lung, liver, kidney, and spleen, derived from all xenograft mouse models (data not shown).

Cetuximab plus regorafenib combination inhibits tumor growth in an orthotopic human colorectal cancer xenograft

An orthotopic colorectal cancer model with HCT116 colorectal cancer cells was established, as described in Materials and Methods. Both cetuximab and regorafenib were well tolerated, and no significant loss of animal weight was observed in the group of combined treatment, whereas a significant weight loss occurred in the single-agent treatment groups, compared with the mice weight before treatment. The observed weight loss in these groups was probably caused by the presence of growing tumors and peritoneal metastases (Supplementary Fig. S4). Of interest, combined...
involved in the EGFR pathways have been hypothesized to play a role in resistance to anti-EGFR drugs in colorectal cancer, including activating mutations in \( \text{KRAS} \), \( \text{NRAS} \), \( \text{B-RAF} \), and \( \text{PIK3CA} \), and loss of expression of \( \text{PTEN} \) (13). The overall scenario is complicated by presence of additional genetic mechanisms able to activate the RAS pathway in the absence of molecular alterations affecting RAS or its immediate downstream effectors (30–37). One strategy to overcome the limitations of targeting an individual growth factor receptor such as the EGFR is to combine different drugs that target different growth controlling pathways. In fact, the use of mAbs blocking an individual pathway has been largely limited by the presence of a compensatory feedback loop in other pathways. In our study, to circumvent this compensatory feedback, we have tested cetuximab in combination with regorafenib in human colorectal cancer cell lines with primary or with acquired resistance to the anti-EGFR mAb cetuximab. The combined treatment with cetuximab plus regorafenib shows a synergistic antitumor effect both \textit{in vitro} and \textit{in vivo}, providing the rationale for the clinical development of this combination. These results are consistent with previous reports, which showed that combined inhibition of different growth controlling pathways might potentially exhibit a better therapeutic efficacy compared with inhibition of a single pathway (38–40). In this respect, regorafenib inhibits multiple cell membrane tyrosine kinase receptors that are involved in key processes of cancer development and progression, including angiogenesis (17). Furthermore, regorafenib antitumor activity could be also due in part by its ability to inhibit RAF serine/threonine kinase (41–43).

We have previously shown that a mechanism of acquired resistance to EGFR inhibitors could be the increased secretion of VEGF, suggesting a key role for tumor-induced angiogenesis in the development of anti-EGFR resistance (21). Moreover, treatment with vandetanib, a dual inhibitor of EGFR and VEGFRs, of human EGFR inhibitor-sensitive colorectal cancer cells could delay the onset of cancer cell resistance (21). Bianco and colleagues (44) have shown that human EGFR inhibitor–resistant cancer cells, secrete VEGF and placental growth factor, and express VEGFR-1. Treatment with vandetanib significantly inhibits VEGFR-1 activation, cell proliferation, and migration in these EGFR inhibitor–resistant human cancer cell lines. Martinelli and colleagues (24) have investigated the role of combined treatment with selective

| Table 2. Cetuximab plus regorafenib combination inhibits growth of orthotopic HCT116 colorectal cancer xenografts |
|-------------------------------------------------|-----------------|-------------------------------|-------------------------------|
| Treatment group                              | Tumor volume (mm\(^3\); %) | Cecal tumor weight (g; %) | Incidence of lymph node metastasis |
| CTR                                           | 15,300 (100%)     | 5.1 (100%)                   | 10/10                         |
| Cetuximab                                    | 13,500 (88%)     | 4.9 (82%)                    | 8/10                          |
| Regorafenib                                  | 10,900 (77%)     | 4.2 (82%)                    | 7/10                          |
| Combination                                  | 750 (4.9%)       | 1.9 (19.6%)                  | 0/10                          |

Figure 4. Cetuximab plus regorafenib combination inhibits growth of orthotopic HCT116 colorectal cancer xenografts. HCT116 cells were injected into the cecal wall of nude mice. Two weeks later, the mice were randomly assigned (7 mice each group) to receive daily administration of PBS/0.5% Tween 80 by oral gavage for 5 days a week and i.p. injection of PBS twice a week (control); daily administration of diluent for 5 days a week and i.p. injection of cetuximab 1 mg twice a week; daily administration of regorafenib 10 mg/kg by oral gavage for 5 days a week and i.p. injection of PBS twice a week; or combination of oral regorafenib and i.p. cetuximab. The treatment continued for 3 weeks, and 1 week later mice were killed and necropsied.
anti-EGFR drugs, such as erlotinib or cetuximab, and sorafenib, another multitargeted inhibitor of C-RAF and B-RAF and of all anti-EGFR drugs, such as erlotinib or cetuximab, and sorafenib, with single-agent erlotinib arm (9.7 months; ref. 50).

In the clinical setting, several studies have explored the possibility of combining anti-EGFR drugs such as cetuximab, panitumumab, or erlotinib, with different antiangiogenic drugs, including bevacizumab or sorafenib. The results in unselected non–small cell lung cancer or colorectal cancer patients have been contradictory (45–49). However, the results of a randomized phase II study in 154 advanced non–small cell lung cancer patients that were selected for the presence of activating EGFR gene mutations have recently demonstrated a statistically and clinically relevant increase in the efficacy of the combined treatment with erlotinib plus bevacizumab compared with single-agent standard therapy with erlotinib. Median PFS was significantly longer in the combination arm (16 months) compared with single-agent erlotinib arm (9.7 months; ref. 50).

A difficult question to answer is whether combining anti-VEGF and anti-EGFR mAbs, at least in combination with cytotoxic drug, has definitively proven to be detrimental, or at least not effective in the first line treatment of metastatic colorectal cancer. Two large randomize phase III studies have evaluated the efficacy of adding an anti-EGFR mAb such as cetuximab (CAIRO-2) or panitumumab (PACCE), to an oxaliplatin-containing chemotherapy doublet plus bevacizumab (48, 49). Both studies have shown that the addition of the anti-EGFR mAbs does not improve efficacy. The possibility of a negative interaction between bevacizumab and anti-EGFR Abs or of a negative interaction when the two Abs and chemotherapy are combined cannot be ruled out, although no mechanisms behind such potential interactions are known. Although these studies have demonstrated a detrimental effect of the combine treatment of cetuximab with bevacizumab in addition to chemotherapy in metastatic colorectal cancer, in our study we have explored the antitumor activity of cetuximab in combination with a different antiangiogenic drug such as regorafenib. Although bevacizumab is an mAb directed against VEGF, regorafenib has a broader spectrum of activity blocking different tyrosine kinase receptors that are potentially involved in the mechanisms of resistance to cetuximab. This may explain the synergistic effect that we have found in this study.

In summary, the present study provides experimental evidence that the combined treatment with anti-EGFR drugs, such as cetuximab, and with a multiple signaling pathway inhibitor, such as regorafenib, could be a potential therapeutic strategy to investigate in a clinical setting for overcoming intrinsic or acquired resistance to EGFR inhibitors in colorectal cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Napolitano, B. Rinaldi, F. Ciardiello, T. Troiani
Development of methodology: S. Napolitano, G. Martini, E. Martinelli, M. Donniacuo, D. Vitagliano, F. Ciardiello, T. Troiani
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Rinaldi, E. Martinelli, M. Donniacuo, G. Barra, R. De Palma, F. Menolla, F. Ciardiello, T. Troiani
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. De Palma, F. Menolla, F. Ciardiello, T. Troiani
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Ciardiello
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Grant Support
This research has been supported by a grant from AssociazioneItaliana per la Ricerca sui Cancro (AIRC) and a grant from Ministero dell’Istruzione, Università e Ricerca (MIUR)-PRIN 2010-2011.

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Received January 6, 2015; revised February 19, 2015; accepted March 20, 2015; published OnlineFirst April 2, 2015.

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Regorafenib and Cetuximab Overcome Resistance to Cetuximab


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