Maintenance treatment with cetuximab and BAY86-9766 increase antitumor efficacy of irinotecan plus cetuximab in human colorectal cancer xenograft models.

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Translational Relevance

EGFR targeted monoclonal antibodies (mAbs), such as cetuximab and panitumumab, are a valid therapeutic strategy for metastatic colorectal (CRC) patients. However, only in a subset of patients with metastatic CRC the addition of these mAbs to chemotherapy increases response rate, progression free survival and overall survival. Patients who initially respond to anti-EGFR therapies will relapse in few months due to development of secondary resistance that limits its clinical efficacy. Further understanding of the molecular alterations of relapse to EGFR blockade is extremely relevant to develop novel therapeutic strategies. In this study, we have demonstrated that in xenograft models, using three human CRC cell lines that are highly dependent on the EGFR pathway, the use of maintenance combined treatment with cetuximab plus MEK 1/2 inhibitor after an induction therapy of irinotecan plus cetuximab, is effective in preventing and/ or overcoming acquired resistance to anti-EGFR inhibitor and in prolonging mice survival.
Abstract

**Purpose:** The use of cetuximab, in the treatment of metastatic colorectal cancer (CRC) is limited by development of resistance.

**Experimental design:** We have investigated in three models of highly EGFR dependent CRC xenografts, the effect of maintenance therapy with different kinase inhibitors alone or in combination with cetuximab, after cytotoxic treatment induction with irinotecan plus cetuximab.

**Results:** SW48, LIM 1215 and GEO CRC cell lines were engrafted into nude mice and treated for three weeks with irinotecan and/or cetuximab. The combined treatment induced a significant reduction of tumor size. A subsequent experiment was performed in all three xenograft models in which after an induction treatment with irinotecan plus cetuximab, mice were randomly assigned to one of the following treatments: control, cetuximab, regorafenib, a selective PIK3CA inhibitor (PIK3CAi), a selective MEK inhibitor (MEKi) and/or the combination of each inhibitor with cetuximab. The cetuximab plus MEKi treatment determined the best antitumor activity with suppression of tumor growth. This effect was prolonged for 13-15 weeks after cessation of therapy and was accompanied by prolonged survival. Antitumor activity was accompanied by inhibition of MAPK and MEK-pathways. Moreover, in the cetuximab plus MEKi treated SW48 xenograft group, *KRAS* mutations as a mechanism of acquired resistance were detected in 25% of cases compared to 75% *KRAS* mutations in the MEKi treated group.

**Conclusions:** A possible strategy to prevent and/or overcome resistance to anti-EGFR inhibitors in metastatic CRC is a maintenance therapy with cetuximab plus MEKi after an initial treatment with irinotecan plus cetuximab.
Introduction

Colorectal cancer (CRC) is the third most common cancer in Western world with 1.2 million of new cases expected worldwide every year (1). The knowledge of colon cancer biology have led to development of biological agents, targeting variable steps in carcinogenesis and metastases, that have been incorporated in the metastatic CRC treatment strategies (2). Thanks to targeted therapeutic options such as the monoclonal antibodies (mAbs) cetuximab and panitumumab, which target the extracellular domain of the Epidermal Growth Factor Receptor (EGFR), the overall survival of patients at metastatic stage has been improved. However, only 10% of objective response rates have been obtained when these drugs are used as monotherapy in unselected patients with chemotherapy-refractory metastatic CRC, suggesting that there is intrinsic or primary resistance to this targeted therapy (3-4).

It is now well established that primary resistance is associated with mutations of genes belonging to RAS pathway (5). In particular, with the extended RAS analysis that covers exons 2,3,4 mutations for \textit{KRAS} and \textit{NRAS} genes the benefit of anti-EGFR mAbs to first-line FOLFIRI chemotherapy for the all-\textit{RAS} wild-type patients has been translated in a further improvement of overall survival (6-10). Unfortunately, even \textit{RAS} wild-type patients can be resistant to anti-EGFR therapies indicating that additional genetic alterations in genes implicated in the EGFR signaling network can be involved in the primary resistance. In fact, deregulation of other effectors of the EGFR signaling cascade, such as mutations in \textit{BRAF} or \textit{PIK3CA} genes, loss of PTEN expression and amplification of \textit{KRAS} are also to be thought to affect primary response to EGFR blockade (7,11-14).

Even in metastatic CRC patients, who initially respond to anti-EGFR therapies, the antitumor efficacy is transient and does not last in median more than 10 to 12 months, after which acquired resistance occurs. The most common molecular mechanisms that are responsible for acquired resistance are genetic alterations of the \textit{KRAS}, \textit{NRAS} and \textit{BRAF} genes (15-18).
In the absence of alteration in RAS or its immediate downstream effectors, other mechanisms have been involved in the activation of the EGFR pathway. Genetic aberrations in receptor tyrosine kinase such as HER2 and MET have been shown to bypass EGFR signaling and activate the MAPK cascade and, therefore to confer acquired resistance to anti-EGFR therapies (19-22).

As with HER2 and MET, alterations to EGFR can contribute to acquired resistance to anti-EGFR (23-25). In a model of colon cancer cell line with acquired resistance to cetuximab, sequencing analysis of the EGFR gene revealed a missense mutation (S492R), which was shown to responsible of cetuximab resistance (23).

It has been demonstrated that another mechanism of acquired resistance to EGFR inhibitors could be an increased secretion of VEGF, suggesting a key role for tumor-induced angiogenesis in the development of anti-EGFR resistance (26-27).

The plethora of alterations that are responsible of both primary and acquired resistant mechanisms to anti-EGFR inhibitors in the treatment of metastatic CRC, biochemically converge in to two “critical points”: MEK-ERK and PIK3CA-AKT pathways. Based on this evidence, we hypothesized that inhibiting simultaneously these survival pathways could render more difficult for cancer cells to escape EGFR blockade. Moreover, if these survival routes are blocked without allowing the tumor to escape from the initial treatment, the time required to develop resistance could be delayed.

We performed an in vivo study using human CRC cell lines highly sensitive to EGFR inhibitors, in order to evaluate which maintenance treatment with different inhibitors downstream of the EGFR pathway, could be able to prevent and/or delay the onset of resistance after an induction treatment with cetuximab plus irinotecan. Among different maintenance treatments we selected three inhibitors, such as regorafenib, GDC-0941 (a selective PIK3CA inhibitor), BAY86-9766 (a selective MEK 1/2 inhibitor) alone or in combination with cetuximab.
Regorafenib is an oral multikinase inhibitor, that acts on angiogenic (VEGFR-2, VEGFR-3, and PDGFR), oncogenic (KIT, PDGFR, and RET), and RTKs involved in the tumor microenvironment and progression (PDGFR-β, FGFR). (28-30). Recently, regorafenib has been approved for the treatment of metastatic CRC patients who failed all available therapies (31).
Materials and Methods

Drugs. Cetuximab, an anti-EGFR human-mouse chimeric monoclonal antibody was kindly provided by Merck Serono Italy (Rome, Italy). GDC-0941 (a selective PIK3CA inhibitor) was generously given by Genentech Inc. (San Francisco, CA, USA). BAY86-9766 (a selective MEK 1/2 inhibitor) and regorafenib were kindly provided by Bayer Pharma Italy (Milan, Italy). Irinotecan was obtained from the pharmacy of the Second University of Naples (Naples, Italy).

Cell Lines. The human SW48 (KRAS, NRAS, BRAF and PIK3CA wild type), colon cancer cell line was obtained and authenticated from IRCCS “Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova” Italy. The human GEO [KRAS mutation (G12A); NRAS, BRAF, and PIK3CA wild type] colon cancer cell line was kindly provided by Dr. N. Normanno (National Cancer Institute, Naples, Italy). The human LIM 1215 (KRAS, NRAS, BRAF and PIK3CA wild type), colon cancer cell line was obtained from Dr.ssa Di Nicolantonio at Candiolo National Cancer Institute (Candiolo, Italy) and authenticated from IRCCS “Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova” Italy. GEO cell lines were grown in DMEM (Lonza, Cologne, Germany) whereas SW48 and LIM 1215 cells were grown in RPMI-1640 (Lonza), supplemented with 10% fetal bovine serum (FBS) (Lonza), 1% penicillin/streptomycin (Lonza).

Tumor xenografts in nude mice. Four- to six-week old female balb/c athymic (nu+/nu+) mice were purchased from Charles River Laboratories (Milan, Italy). Mice were maintained in accordance with the institutional guidelines of the Second University of Naples Animal Care and Use Committee. In brief, 2.5 x 10^6 SW48, 1 x 10^6 GEO and LIM 1215 cells had been suspended in 200 µl of matrigel (BD Biosciences, Milan, IT) and injected subcutaneously into female nude mice. When tumors reached a mean volume of 200-400 mm³, animals were distributed into four groups consisting of 10 animals per group. Irinotecan was administered intraperitoneally (i.p.) (100mg/kg) once a week for three weeks. Cetuximab at the dose of 1 mg was injected intraperitoneally twice a
week for three weeks. Subsequently, groups of 70 mice each were injected subcutaneously with SW48, GEO or LIM 1215 human colon cancer cell lines. After two weeks, animals were treated with irinotecan (100mg/kg once a week, i.p.) plus cetuximab (1mg twice a week, i.p.). At the end of three weeks of therapy, defined as induction treatment mice were randomized into 8 groups (n=10 mice per group) as shown in Figure 2. Cetuximab (1 mg twice a week, i.p.) was dosed i.p., whereas BAY 86-9766 (25mg/kg every day for 5 days a week), GDC-0941 (75mg/kg every day for 5 days a week) and regorafenib (10mg/kg every day for 5 days a week) were given by oral gavage. The maintenance treatment was continued for 8 weeks and afterwards animals were followed for additional 17 weeks (follow-up period). The mice body weights were monitored and tumors were measured with a caliper using the following formula: π/6 x larger diameter x (smaller diameter)^2. At the end of maintenance treatment, five animals per group were sacrificed and four tumor samples were collected for multiple gene mutation analysis by next-generation sequencing, whereas one was used for Western Blot analysis.

**Immunoblotting.** Tumor sample were harvested from euthanized mice, cut into 20-25 mm³ pieces and frozen at -80 C in RNA later. Subsequently frozen samples were homogenised in RIPA lyses buffer (0,1% sodium dodecylsulphate (SDS), 0,5% deoxycholate, 1% Nonidet, 100mM NaCl, 10 mM Tris-HCl (pH 7,4)) containing a protease inhibitor cocktail (Hoffmann-La Roche, Basel, Switzerland), 0,5 mM dithiotritol, and 0,5% phenylmethyl sulphonyl fluoride. Tissue lysates were clarified by centrifugation at 14,000rpm for 10 min a 4 C °. Protein lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad, Munich, Germany), were subjected to Western blot, as previously described (32). Desired proteins were probed with corresponding antibodies. EGFR and phospho-EGFR monoclonal antibody, MET and phospho-MET monoclonal antibody, IGFRβ and phospho-IGFRβ monoclonal antibody, p44/42 MAPK polyclonal antibody, phospho-p44/42MAPK monoclonal antibody, MEK1/2 and phospho-MEK1/2 monoclonal antibody, AKT polyclonal antibody, phospho-AKT monoclonal antibody (#4060), were
from Cell Signaling (Beverly, MA, USA). Monoclonal anti-α-tubulin antibody (T8203) was from Sigma Chemical Co. (St. Louis, MO, USA). The following secondary antibodies from Biorad (Hercules, CA, USA) were used: goat anti-rabbit IgG and rabbit anti-mouse IgG. Immunoreactive proteins were visualized by enhanced chemiluminescence. (ECL plus, Thermo Fisher Scientific, Rockford, IL, USA). Each experiment was done in triplicate.

**Multiple gene mutation analysis by next-generation sequencing.** Tumour sample were harvested from euthanized mice, cut into 20-25 mm³ pieces, formalin-fixed, paraffin-embedded and analyzed with the Ion AmpliSeqTM Colon and Lung Cancer Panel (Life Technologies) using Ion Torrent semiconductor sequencing as previously described (33). In particular the panel contains primer pairs to analyze over 500 known mutations and eventually novel mutations in 87 hotspot regions of the following 22 genes: **ALK, EGFR, ERBB2, ERBB4, FGFR1, FGFR2, FGFR3, MET, DDR2, KRAS, PIK3CA, BRAF, AKT1, PTEN, NRAS, MAP2K1, STK11, NOTCH1, CTNNB1, SMAD4, FBXW7, TP53** (33).

**Statistical analysis.** The statistical analyses of *in vivo* data were carried out using the SPSS package (version 21.0 for Windows, SPSS Inc., USA). The Student’s *t* test was used to evaluate the statistical significance of differences between treatment effects. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. All the tests were two-sided, with *P* value of <0.05 considered to indicate statistical significance.
Results

The combination of irinotecan and cetuximab lead to enhanced antitumor efficacy in CRC xenograft models.

In the present study, we have selected three human colorectal cancer cell lines (SW48, GEO and LIM1215) that are sensitive to EGFR inhibition (33-35). In particular the SW48 and LIM 1215 cell lines function as a relevant model for metastatic CRC patients that would receive cetuximab treatment as neither of cell line has genetic alterations that are known to be associated with intrinsic resistance to anti-EGFR therapies (KRAS, BRAF, and PIK3CA) (7; 34-36). On the contrary, GEO cells display a different genetic profile, harboring KRAS codon 12 mutation (34). Despite KRAS gene mutation, previous studies from different laboratories, including our own, demonstrated that this CRC cell line is one of the most sensitive to the in vitro and in vivo antitumor activity of cetuximab treatment (34-36).

Mice were treated intraperitoneally for three weeks with cetuximab, irinotecan and their combination. As shown in Figure 1, both agents determined in all three models a tumor growth inhibition that was more pronounced in the cetuximab arm reaching a statistical difference compared with control (p< 0.05). In addition, the combination of irinotecan plus cetuximab resulted in tumor regression during the treatment period (Figure 1). The combined treatment was well tolerated with no weight loss or other signs of acute or delayed toxicity (data not shown).

Trial design and treatment plan.

The mechanisms of acquired resistance to EGFR blockade that have been confirmed clinically range from genetic alterations in members of the EGFR-RAS-RAF-MEK pathway to activation of pathways driven by other receptor tyrosine kinases (RTKs), such as MET, ERBB2 or VEGFR1 (17-26). A possible strategy to overcome acquired resistance is to prevent it from emerging by using
combination treatment as first line targeted therapy rather than after the patient has progressed on therapy. Based on this assumption, we designed an *in vivo* study in which each of three CRC cell lines described above was injected subcutaneously into right flank of a group of 80 female nude mice. After two weeks from the injection, mice were treated for three weeks with the combination of irinotecan (100 mg/kg once a week) plus cetuximab (1 mg twice a week). These doses were chosen, on the basis of the previous experiment, as the optimal doses that were able to determine tumor growth inhibition after three weeks of treatment without relevant side effects (Figure 1). At the ends of this induction treatment, mice were randomized into 8 group (10 per group) and treated for eight weeks with several kinase inhibitors, such as regorafenib, GDC-0941 (a selective PI3CA inhibitor), BAY86-9766 (a selective MEK 1/2 inhibitor) alone or in combination with cetuximab (Figure 2A). After this maintenance treatment mice were followed for another 17 weeks.

**Antitumor efficacy of irinotecan plus cetuximab followed by maintenance treatment in human CRC xenograft models.**

On week 13, at the end of maintenance treatment, all mice treated with cetuximab and xenografted with SW48 and GEO cells reached the maximum allowed tumor size of 2000 mm$^3$ unlike those xenograft with LIM 1215 (Figure 2 B-C-D and Figure 3). Among the single agent treatments, the group treated with MEK inhibitor (MEKi) showed the greatest tumor growth inhibition in all three xenografts. In fact, the growth rate of tumors treated with regorafenib was similar to those treated with cetuximab regardless of cell lines injected into the mice (Figure 3). A similar trend was observed for the group treated with the PI3K inhibitor (PI3Ki) in GEO xenograft with mean tumor volume of 1800 mm$^3$ and tumor growth inhibition of 30%. On the contrary in the SW48 and LIM 1215 xenografts, treatment with PI3Ki produced a 61% and 37% of tumor growth inhibition, respectively with 90% and 70% of mice still alive at the end of therapy. Moreover, SW48, GEO and LIM 1215 tumor growth was significantly inhibited in mice treated with MEKi as
compared with cetuximab–treated mice. In fact MEKi treatment induced a 92 %, 84% and 83% of tumor growth inhibition in SW48, GEO and LIM1215, respectively (Figure 3).

In the combined treatment groups no benefit was shown in terms of tumor growth inhibition by adding PI3Ki to cetuximab as compared to single agent treatments in all xenograft models (Figure 3). Moreover, even if the combination of cetuximab plus regorafenib caused a potent cooperative antitumor activity in all three xenograft models, the best antitumor activity has been obtained by combining cetuximab plus MEKi. In fact, this combination caused an almost complete suppression of tumor growth in SW48, GEO and LIM 1215 with a mean tumor volume of 22 mm$^3$, 140 mm$^3$ and 22mm$^3$ respectively. Moreover, in this treatment group, animal with no evidence of tumors were more than double as compared to single MEKi treatment.

We observed animals for others 17 weeks (follow-up period) from the end of treatment to evaluate which treatment could determine a more sustained and prolonged tumor growth control. In the combined cetuximab plus regorafenib group, tumor started to regrowth immediately after the cessation of treatment. However, even if more prolonged tumor inhibition was seen in the MEKi groups, 3-5 weeks after cessation of treatment the SW48, GEO and LIM 1215 tumor growth rate was comparable with growth rate of tumors in cetuximab treated mice. On the contrary, the antitumor activity of the combined cetuximab plus MEKi treatment was still maintained after 13-15 weeks after the cessation of therapy, eventually reaching a growth rate comparable with cetuximab treated tumors only at the end of the follow-up period (Figure 3).

**Antitumor response in CRC xenograft models.**

For monitoring tumor responses to therapy, we measured volumetric changes and used an arbitrary classification method partially based on clinical research (19) : complete response (RC), was defined as no clinical evidence of tumor when mice were sacrificed; partial response (RP), was defined as a decreased of at least 30 % in tumor volume with respect to the baseline tumor volume;
progression disease (PD), was defined as an increase of at least 20% in the tumor volume with respect to the baseline tumor volume; and finally, responses that were neither sufficient reduction to categorize regression nor sufficient increase to categorize progression were considered as stable disease (SD). Based on this criteria, Figure 4 shows a waterfall plot of the effect of the three best therapeutic regimens on tumor growth after 8 weeks of therapy.

The proportion of mice that achieved a complete response was similar between MEKi and cetuximab plus regorafenib treatments in all three xenograft models, ranging from 10 to 20% (Figure 4). In the group treated with cetuximab plus MEKi the percentage of RC was more than double compare to the group treated with single agent MEKi, ranging from 30 to 40%, suggesting that in this group there is the highest percentage of complete responses (Figure 4). No partial responses were identified in the cetuximab plus regorafenib group xenograft with SW48 cells. Moreover, in mice xenograft with GEO and LIM 1215, cetuximab plus regorafenib treatment determined the lowest percentage of partial responses with only 1 mouse out of 10 (10%) and 2 out of 10 (20%) achieving a RP, respectively. The treatment with MEKi alone or in combination with cetuximab determined a similar partial response rate in GEO xenograft model. The best disease control rate (RC+PR+SD) was observed in the cetuximab plus MEKi treatment group. In fact, in all three xenograft models no evidence of tumor progression has been detected with 100% of disease control rates (Figure 4). The delayed tumor growth in the cetuximab plus MEKi-treated group was accompanied by a prolonged survival that was significantly different compared with cetuximab or with MEKi treated groups (Figure 5). In particular, median overall survival was 30 months (27.5-33.2; 95% Confidence Interval, 95% CI) in the cetuximab plus MEKi arm for all xenograft group compared with 10 (7.9-12.1; 95% Confidence Interval, 95% CI), 9 (6.9-11.06;95% Confidence Interval, 95% CI) and 12 (10.4-13.5;95% Confidence Interval, 95% CI) months in the cetuximab arm and 25 (21.9-28.1;95% Confidence Interval, 95% CI), 24 (19.3-28.6;95% Confidence Interval,
95% CI) and 25 (21.9-28; 95% CI) months in the MEKi arm for SW48, GEO and LIM 1215, respectively (Figure 5).

**Distinct genetic events may drive acquired resistance to cetuximab in the CRC xenografts.**

It has been demonstrated that CRC cell lines with acquired resistance to cetuximab or panitumumab showed the concomitant presence of different genetic mutations, suggesting that the presence of resistant clones may confer resistance to the drugs (17). For this reason at the end of the maintenance treatment, four animals per group from the SW48 xenograft model were sacrificed and tumor samples were collected and analyzed with the Ion AmpliSeq™ Colon and Lung Cancer Panel. As control we used four mice engrafted with SW48 that had not undergone to any type of treatment from the first in vivo experiment. A 2% sensitivity threshold was set for this next generation sequencing panel, following the results of a validation study (33; 37). As shown in Figure 6A, no mutations were found in 26 of 32 cases analyzed for 22 genes. Among the 22 genes, mutations were only found in 3 genes. Mutations profiling revealed molecular alterations in KRAS, BRAF and PIK3CA (Figure 6A). The most frequent mutated gene was KRAS harboring a common mutation in exon 2 (i.e. codon 13). One rare variant of BRAF mutations was observed in exon 15 of BRAF (pV600K) that co-existed with KRAS mutation p.G13D. Moreover, two rare mutations in PIK3CA gene were found one in exon 20 (p. H1047Y) and another in exon 9 (p. Q546R) (Figure 6B).

No mutations were found in the control, cetuximab, cetuximab plus PI3Ki and cetuximab plus regorafenib treated groups (Figure 6A and B). Three out of four (75%) mice treated with MEKi harbored KRAS mutation with one case having a co-existed mutation with BRAF. On the contrary, only one mouse out of four (25%) treated with cetuximab plus MEKi harbored a mutation in KRAS. PIK3CA mutations were found in one out of four (25%) mice treated with PI3Ki and one out of four (25%) mice treated with regorafenib. However the low frequency of PIK3CA mutations does not
allow us to draw any conclusion on the relevance of this gene mutation in the potential acquisition of resistance (Figure 6B).

**Effects of cetuximab plus MEKi treatment on EGFR-dependent intracellular signaling pathways in CRC xenograft models.**

To understand whether the synergistic anti-tumor activity obtained by the combined treatment with cetuximab and MEKi was due to a more effective inhibition of EGFR downstream effectors, tumors were collected at the end of the maintenance treatment from mice engrafted with the SW48, GEO and LIM 1215 cell lines. As shown in Figure 6C, in tumor specimens treated with cetuximab, no change in expression of pEGFR, pMEK and pMAPK was observed whereas in tumor specimens treated with MEKi the phosphorylation of these proteins was partially inhibited. The combined treatment with cetuximab and MEKi substantially inhibited phosphorylation of EGFR, MEK and MAPK compared to single agent treatments (Figure 6C). Moreover, no change in expression of pAKT was observed among the different treatment groups.

Previous studies have demonstrated that activation of survival pathways such as MET and IGFR-1 could be responsible for the acquired resistance to anti-EGFR inhibitors (21-22, 35, 38). Interestingly, whereas IGFR-1 and MET proteins were expressed in all tumors the activation of these proteins, as detected by the presence of pIGFR-1 and pMET was observed only after treatment (Figure 6C).
Discussion

The understanding of molecular features of tumors has revealed that cancer genomes are highly heterogeneous especially in the case of metastatic dissemination (39). These characteristics may explain why treatments with individual drugs, such as anti-EGFR inhibitors, have limited effectiveness. In particular, the clinical limitation of the two monoclonal antibodies, cetuximab and panitumumab, in the treatment of metastatic CRC due to emergence of secondary resistance prompted us to study how this problem could be potentially tackled. The idea of this in vivo study has been founded on two considerations: first that mechanisms of acquired resistance to anti-EGFR inhibitors in the treatment of metastatic CRC are extremely heterogeneous ranging from genetic mutations of the EGFR-RAS-RAF-MEK to activation of independent pathways driven by other tyrosine kinases receptors (RTKs) (40-41). This suggests that the concomitant blockade of signaling nodes that could confer resistance to anti-EGFR inhibitors might be a possible strategy to overcome it. Second, that if these signaling pathways are blocked from the beginning without offering the tumor the possibility to first escape the initial treatment, the time required to develop resistance could be extended. As a proof of concept, we used three CRC cell lines that are highly sensitive to EGFR inhibitions, grown as tumor xenograft in nude mice. We treated mice for three weeks with a combination of irinotecan plus cetuximab that is considered a possible strategy to treat metastatic CRC patients harboring no mutations in RAS genes as first line therapy (10). Before tumor started to regrowth and eventually possible resistance mechanisms to targeted agents will occur, mice were randomized to maintenance treatments with several inhibitors, such as regorafenib, PI3Ki, MEKi alone or in combination with cetuximab.

The results of this study suggest that the combined treatment with cetuximab plus MEKi, after an induction therapy of irinotecan and cetuximab, is able to prevent and/ or overcome the resistance to anti-EGFR inhibitors. In particular, maintenance treatment for eight weeks with cetuximab plus
MEKi after a three weeks induction treatment with irinotecan plus cetuximab is highly effective in increasing tumor growth inhibition with 60 to 80% major tumor responses in the three CRC cell models that were studied and with 2 out of 10 mice cured in the combination cetuximab plus MEKi. This antitumor activity was translated in a better antitumor efficacy since median overall survival was significantly increased from 10 to 12 weeks in mice treated with cetuximab alone to approximately 30 weeks in mice treated with cetuximab plus MEKi.

In agreement with these results, some preclinical and clinical studies have shown that combination therapy is able to delay the onset of resistance to BRAF inhibitors in the treatment of metastatic melanoma (42-43). In particular, it has been shown that the combination of BRAF inhibitors and MEK inhibitors is a therapeutic option to delay the onset of acquired resistance when administrated in the first line setting in metastatic melanoma but does not necessarily reverse established resistance to BRAF inhibitors.

These results are in agreement with previous results obtained by our group and by others laboratories in which it has been clearly demonstrated a central role of the MEK-MAPK pathway as a possible resistance mechanism to anti-EGFR therapy (15, 17). In particular, in a previous study we have demonstrated that the combined treatment of cetuximab with selective MEK inhibitors is able to overcome the resistance to cetuximab in a panel of CRC with both primary and acquired resistance to anti-EGFR inhibitors (34-35).

Misale et al. have demonstrated that in CRC cell lines with acquired resistance to cetuximab, point mutations and/or amplifications of KRAS triggered to loss of sensitivity to cetuximab (15). In the presence of cetuximab, KRAS activity was not depleted for resistant cell lines, and this was concomitant with maintained phosphorylation of MEK and ERK. Moreover, pharmacological inhibition of MEK when combined with cetuximab also sensitized the resistant cells to anti-EGFR therapy (15).
In a subsequent study, the same group has demonstrated the emergence of polyclonal \textit{KRAS}, \textit{NRAS}, and \textit{BRAF} mutations in CRC cells with acquired resistance to EGFR blockade (17). Regardless of the genetic alterations resistant cells consistently displayed MEK and ERK activation, which persisted after EGFR blockade. Inhibition of MEK1/2 alone failed to impair the growth of resistant cells \textit{in vitro} and \textit{in vivo} (17). Indeed, concomitant pharmacological blockade of MEK and EGFR induced prolonged ERK inhibition and severely impaired the growth of resistant tumor cells (17).

One of the most common molecular mechanism that drive secondary resistance to anti-EGFR therapy in metastatic CRC patients is the acquisition of new genetic alteration of \textit{KRAS}, \textit{NRAS} and \textit{BRAF} genes (13, 16-18). In particular, under the pressure of a selective treatment, resistant subclones preexisting in the initial tumor cell population will be selected and will be responsible of the resistance. These cancer cell populations cannot be detected by gene sequencing but could be found with more sensitive approaches such as NGS. In particular in our experiment, mutation profiling revealed molecular alterations only in \textit{KRAS} and \textit{BRAF} following treatment with different inhibitors. Furthermore, the highest frequency of mutations in \textit{KRAS} gene was found in the group treated with MEKi alone, whereas it decrease in the cetuximab plus MEKi combined treatment group. These results suggest that the combination treatment with cetuximab and MEKi prevents or delays the selection of clones that carry the mutation in genes responsible of acquired resistance to anti-EGFR inhibitors.

Several groups have tried to address the issue if the resistant clones are present before therapy is given and selected for under pressure of drug treatment or if there are truly acquired. In particular, Misale and colleagues demonstrated that \textit{KRAS} G13D gene mutation and \textit{KRAS} amplification was found also in the parental population and not in the cetuximab-resistant cells, suggesting that the acquired resistance could be a selection of clones that could have a preferential advantage under cetuximab treatment (15).
There is some evidence, though, that mutations that lead to resistance can be acquired during drug treatment. In fact, similar to our results, Misale et al. were unable to detect the LIM1215 cetuximab resistant \textit{KRAS} G12R mutation in the LIM1215 parental cells implying that this mutation was acquired during treatment (15). Moreover the EGFR S492 gene mutation that was demonstrated responsible for the acquisition of cetuximab resistance was not found in metastatic CRC patients before anti-EGFR treatment (33, 44).

Another important finding in the present study is the detection of the activation of alternative survival pathways, such as the activation of MET and IGFR-1 signaling under the pressure of selective drugs, as a mechanism of acquired resistance to anti-EGFR inhibitors. In particular, we found that biochemical activation of these pathways could be detected before of clinical progression was documented by the increase in tumor size in mice.

Several reports indicate that amplification or constitutive activation of the \textit{MET} gene confers resistance to EGFR blockade (21-22,35). Stimulation of the MET receptor by its ligand could confer resistance to cetuximab and panitumumab in CRC cells and xenografts, but this could be overcome by pharmacological inhibition of MET or by and silencing of the \textit{MET} gene (21-22, 35). Recently, a study on seven CRC patients that progressed following initial response to cetuximab found that three harbored \textit{KRAS} mutations and three had amplification of the \textit{MET} gene in the post-treatment biopsy (21). Amplification of the \textit{MET} gene in these patients correlated with increased expression of the MET protein. Moreover, in this study it was demonstrated that \textit{MET} gene amplification was present on the plasma prior to computer tomography scan detection of disease progression (21).

In this respect, our research group has contributed to elucidate the potential role of IGFR-1 and MET in the resistance to anti-EGFR inhibitors in lung cancer (38). Increase in phosphorylated, activated IGFR-1 and MET growth factor receptors was observed in CALU-3 lung adenocarcinoma.
cells with acquired resistance to four different tyrosine kinase inhibitors, including erlotinib, gefitinib, sorafenib and vandetanib (38).

In conclusion, the acquired resistance to anti-EGFR inhibitors can be defined as a consequence of a perturbation in a system in which the initial equilibrium is based on cell that are addicted to EGFR signaling. The evidence that most of the mutations that emerge upon treatment involve genes that are direct members of the EGFR pathways indicate that to escape the perturbations, the cells must settle on a new balance which is based again on EGFR signaling. This is supported by this study that, in agreement with data coming from other groups (17,34,38), suggests that the plethora of alterations that emerge as a mechanism of acquired resistance to anti-EGFR inhibitors in the treatment of metastatic CRC patients could converge on the activation of the RAS-RAF-MEK-MAPK pathway. Therefore, an effective therapeutic strategy could be to add in the treatment sequence for EGFR-dependent CRC the use of selective MEK inhibitors in combination with anti-EGFR drugs following initial treatment with chemotherapy plus anti-EGFR drugs.
Acknowledgments

This research has been supported by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC) and a grant from Ministero dell’ Istruzione, Università e Ricerca (MIUR)-PRIN 2010-2011.
References


38) Morgillo F, Cascone T, D’Aiuto E, Martinelli E, Troiani T, Saintigny P, et al. Antitumour...


Figure Legends

Figure 1. Effects of cetuximab in combination with irinotecan on CRC tumor xenografts. Mice were injected subcutaneously in the dorsal flank with SW48, GEO and LIM 1215 cell lines as described in the “Materials and Methods” section. After two weeks (average tumor size 200-400 mm³) mice were treated intraperitoneally for three weeks with: PBS (phosphate-buffered saline) control, cetuximab (1 mg twice a week), irinotecan (100mg/kg once a week) and their combination. Each group consisted of 10 mice. Tumor volumes were measured three times a week. Student’s t test was used to compare tumor sizes among different treatment groups and control untreated mice at week five following CRC cell injection. Irinotecan+cetuximab versus Control and versus Irinotecan (* p < 0.05 for all three xenograft models).

Figure 2. Effect of maintenance treatment with several kinase inhibitors, alone or in combination with cetuximab, after induction cytotoxic therapy with irinotecan plus cetuximab in CRC xenograft models. A)Treatment schedule. SW48, GEO and LIM 1215 cells were injected s.c.into the left flanks of nude mice. After two weeks of subcutaneous injection mice were treated with Irinotecan (100mg/kg once a week) in combination with cetuximab (1 mg twice a week) for three weeks (induction treatment). Afterwards mice were divided randomly into 8 groups of 10 mice and treated for 8 weeks (maintenance treatment). CTR, control; MEKi, MEK inhibitor or BAY86-9766 (25mg/kg every day for 5 days a week, by oral gavage); PI3Ki, PI3K inhibitor or GDC-0941 (75mg/kg every day for 5 days a week, by oral gavage); regorafenib (10mg/kg every day for 5 days a week, by oral gavage). Black boxes, cetuximab treatment days; blue boxes, MEKi treatment days; green boxes, PI3Ki treatment days; yellow boxes, regorafenib treatment days. B-D) Antitumor activity of maintenance treatment in SW48, GEO and LIM 1215 tumor bearing mice. The indicated cancer cell lines were grown as subcutaneous tumor xenografts in nude mice and treated with different drugs as indicated above. The mean data are present. Tumor growth
curves were calculated based on three times a week tumor measurements during the treatment period and after seventeen weeks of observation after termination of therapy.

**Figure 3. Antitumor efficacy of irinotecan plus cetuximab treatment followed by maintenance treatment in human colon SW48, GEO and LIM 1215 xenografts.** Mice injected subcutaneously with indicated colon cancer cell lines where treated with irinotecan plus cetuximab from week 2 to week 5. Subsequently from week 5 to week 13 were randomly divided in seven groups and treated with indicated drugs. Tumor volume was measured three times per week until week 30. Animals were sacrificed when tumors achieved 2,000 mm$^3$ in size. Values are expressed as mean for each group over the given time frame. Abbreviations: CTR, Control, MEKi, MEK inhibitor; PI3Ki, PI3K inhibitor; A, median tumor volume (mm$^3$); B, alive mice/total mice; C, number of mice with clinical complete remission.

**Figure 4. Best antitumor response in mCRC xenograft after the end of maintenance treatment.** Waterfall plot of response to cetuximab plus regorafenib (cetuximab+regorafenib), MEKi (MEK inhibitor) and cetuximab plus MEKi (cetuximab + MEKi) normalized against tumor volume at baseline. Each bar represents the change of tumor volume of individual mice. Cases experiencing Complete Response, Stabilization, Partial Response and Disease Progression are shaded in light blue, violet, green and pink respectively.

**Figure 5. Effects of maintenance treatment on the survival of SW48, GEO and LIM 1215 tumor bearing mice.** Experimental design is described in the legend of figure 2. Mice were monitored for survival until 30 weeks following tumor cell injection. Differences in animal survival among groups were evaluated by use of the Mantel Cox logrank test. MEKi versus control, Cetuximab+MEKi versus control, cetuximab + PI3Ki versus control, cetuximab + regorafenib versus control (** p < 0.05 for all three xenograft models); PI3Ki versus control (** p < 0.05 in
SW48 and LIM 1215 xenograft models); regorafenib versus control (** \( p < 0.05 \) in SW48 and LIM 1215 xenograft models); regorafenib versus control (* \( p < 0.05 \) in GEO xenograft model).

Figure 6. Gene mutations analysis by Next Generation Sequences and effect of cetuximab plus MEKi on intracellular signaling pathways of SW48 xenograft model. A-B) Genetic alterations. 4 animals per group of SW48 xenograft model were scarified at the end of “maintenance therapy”. Tumor samples were collected and analyzed with the Ion AmpliSeqTM Colon and Lung Cancer Panel (Life Technologies) using Next Generation Sequencing, as described in “Materials and Methods”. List of gene and molecular alterations have been described. C) Analysis of intracellular signalling pathways by Western blotting in the CRC xenograft models. At the end of maintenance treatment one mouse per group treated with cetuximab, MEKi or with their combination was sacrificed. As control we used one mouse that has not undergone to any type of treatment from the first in vivo experiment. Tumour samples was collected and total cell protein extracts were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods. Anti-tubulin antibody was used for normalization of protein extract content.
Figure 1

**SW48**

- CTR
- Cetuximab
- Irinotecan
- Irinotecan+Cetuximab

**GEO**

**LIM 1215**

Tumor Volume (mm$^3$) vs. Weeks for SW48, GEO, and LIM 1215 with different treatment groups.
**Figure 2**

A) Tumor volume (mm$^3$) over time for different treatments. Irinotecan + Cetuximab (1 day) followed by randomization.

B) SW48: Tumor volume (mm$^3$) over time for different treatments. Induction treatment, maintenance treatment, and follow-up period.

C) GEO: Tumor volume (mm$^3$) over time for different treatments. Induction treatment, maintenance treatment, and follow-up period.

D) LIM 1215: Tumor volume (mm$^3$) over time for different treatments. Induction treatment, maintenance treatment, and follow-up period.
### SW48

<table>
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<th>Week 5</th>
<th>Week 13</th>
<th>Week 30</th>
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<td>B</td>
<td>C</td>
<td>A</td>
</tr>
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</tr>
<tr>
<td>Cetuximab</td>
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<td>0</td>
<td>125</td>
</tr>
<tr>
<td>MEKi</td>
<td>210</td>
<td>10/10</td>
<td>0</td>
<td>125</td>
</tr>
<tr>
<td>PI3Ki</td>
<td>210</td>
<td>10/10</td>
<td>0</td>
<td>125</td>
</tr>
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<td>Regorafenib</td>
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<td>10/10</td>
<td>0</td>
<td>125</td>
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<tr>
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### GEO

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### LIM 1215

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<td>C</td>
<td>A</td>
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<td>Cetuximab+ MEKi</td>
<td>305</td>
<td>10/10</td>
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<td>Cetuximab+ PI3Ki</td>
<td>305</td>
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<td>305</td>
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<td>0</td>
<td>105</td>
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</tbody>
</table>
Figure 4

**SW48**

- **Cetuximab+Regorafenib**
  - Mice
  - RC: 20%; PD: 40%; SD: 40%

- **MEKi**
  - Mice
  - RC: 20%; PR: 40%; PD: 40%

- **Cetuximab+MEKi**
  - Mice
  - RC: 40%; PR: 30%; SD: 30%

**GEO**

- **Cetuximab-regorafenib**
  - Mice
  - RC: 10%; PR: 10%; SD: 40%; PD: 40%

- **MEKi**
  - Mice
  - RC: 10%; PR: 40%; SD: 20%; PD: 40%

- **Cetuximab-MEKi**
  - Mice
  - RC: 20%; PR: 40%; SD: 40%

**LIM 1215**

- **Cetuximab-regorafenib**
  - Mice
  - RC: 10%; PR: 20%; SD: 30%; PD: 40%

- **MEKi**
  - Mice
  - RC: 10%; PR: 40%; SD: 10%

- **Cetuximab-MEKi**
  - Mice
  - RC: 30%; PR: 50%; SD: 20%
### SW48

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<tr>
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<tr>
<td>MEKi</td>
<td>25 (21.9 - 28.1)</td>
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<tr>
<td>PI3Ki</td>
<td>18 (13.9 - 22.1)</td>
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<tr>
<td>Regorafenib</td>
<td>12 (7.4 - 16.6)</td>
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<td>30 (27.5 - 33.2)</td>
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<td>19 (14.9 - 23.1)</td>
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### GEO

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<td>MEKi</td>
<td>24 (19.3 - 28.6)</td>
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<td>PI3Ki</td>
<td>11 (9.4 - 12.5)</td>
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<td>Regorafenib</td>
<td>12 (8.9 - 15.0)</td>
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<tr>
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<td>30 (23.9 - 36.0)</td>
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<tr>
<td>Cetuximab+PI3Ki</td>
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<td>23 (18.3 - 27.6)</td>
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### LIM 1215

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### Figure 6

#### A

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<th>Frequency of Genetic Alteration (percentage %)</th>
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<td>MEKI</td>
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<td>Cetuximab+MEKi</td>
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<td>Cetuximab+PI3Ki</td>
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#### B

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<th>Treatment Group</th>
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<tr>
<td>MEKI</td>
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<td>KRAS p.G13D (20.9%)</td>
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#### C

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</table>

![Western Blot Images](SW48, GEO, LIM1215)
Clinical Cancer Research

Maintenance treatment with cetuximab and BAY86-9766 increase antitumor efficacy of irinotecan plus cetuximab in human colorectal cancer xenograft models

Teresa Troiani, Stefania Napolitano, Giulia Martini, et al.

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