Molecular Cancer Therapeutics

EPHA2 Is a Predictive Biomarker of Resistance and a Potential Therapeutic Target for Improving Antiepidermal Growth Factor Receptor Therapy in Colorectal Cancer

Giulia Martini[1], Claudia Cardone[1], Pietro Paolo Vitiello[1], Valentina Belli[1], Stefania Napolitano[1], Teresa Troiani[1], Davide Ciardiello[1], Carminia Maria Della Corte[1], Floriana Morgillo[1], Nunzia Matrone[1], Vincenzo Sforza[2], Gianpaolo Papaccio[3], Vincenzo Desiderio[3], Mariel C. Paul[4], Veronica Moreno-Viedma[4], Nicola Normanno[5], Anna Maria Rachiglio[5], Virginia Tirino[3], Evaristo Maiello[6], Tiziana Pia Latiano[6], Daniele Rizzi[6], Giuseppe Signoriello[7], Maria Sibilia[4], Fortunato Ciardiello[1], and Erika Martinelli[1]

Abstract

The EPHA2 tyrosine kinase receptor is implicated in tumor progression and targeted therapies resistance. We evaluated EPHA2 as a potential resistance marker to the antiepidermal growth factor receptor (EGFR) monoclonal antibody cetuximab in colorectal cancer. We studied activation of EPHA2 in a panel of human colorectal cancer cell lines sensitive or resistant to anti-EGFR drugs. The *in vitro* and *in vivo* effects of ALW-II-41-27 (an EPHA2 inhibitor) and/or cetuximab treatment were tested. Formalin-fixed paraffin-embedded tumor specimens from 82 *RAS* wild-type (WT) metastatic colorectal cancer patients treated with FOLFIRI + cetuximab as first-line therapy in the CAPRI-GOIM trial were assessed for EPHA2 expression by immunohistochemistry and correlated with treatment efficacy. EPHA2 was differentially activated in colorectal cancer cell lines. Combined treatment with ALW-II-41-27 plus cetuximab reverted primary and acquired resistance to cetuximab, causing cell growth inhibition, inducing apoptosis and cell-cycle G1–G2 arrest. In tumor xenograft models, upon progression to cetuximab, ALW-II-41-27 addition significantly inhibited tumor growth. EPHA2 protein expression was detected in 55 of 82 tumor samples, frequently expressed in less-differentiated and left-sided tumors. High levels of EPHA2 significantly correlated with worse progression-free survival [8.6 months; confidence interval (CI) 95%, 6.4–10.8; vs. 12.3 months; CI 95%, 10.4–14.2; $P = 0.03$] and with increased progression rate (29% vs. 9%, $P = 0.02$). A specific EPHA2 inhibitor reverts *in vitro* and *in vivo* primary and acquired resistance to anti-EGFR therapy. EPHA2 levels are significantly associated with worse outcome in patients treated with FOLFIRI + cetuximab. These results highlight EPHA2 as a potential therapeutic target in metastatic colorectal cancer.

Introduction

In the past 20 years, the medical treatment for colorectal cancer has been evolving (1). In particular, the use of new cytotoxic drugs and of molecular targeted agents has conferred a significant improvement in the prognosis of patients with metastatic disease, in which the median overall survival (OS) has increased from 6 months, when only best supportive care was available, to nearly 30 months with the introduction of multiple lines of treatment, that include all active anticancer drugs, such as fluoropyrimidines, irinotecan, oxaliplatin, and molecular targeted agents (antiangiogenesis and antiepidermal growth factor receptor, EGFR, drugs; ref. 2). Approximately 25% of patients with colorectal cancer present metastatic disease at the time of the diagnosis and 40% of newly diagnosed patients will develop metastatic disease during follow-up (3). Despite the advantage on response rate (RR), progression-free survival (PFS), and OS obtained by treating patients with targeted therapies, all patients relapse mostly for the emergence of mechanisms of cancer cell resistance (4). For anti-EGFR monoclonal antibody (mAb) therapy in metastatic colorectal cancer, mutations in genes encoding for EGFR-activated

[1]Medical Oncology, Department of Precision Medicine, Università degli Studi della Campania L. Vanvitelli, Naples, Italy. [2]Department of Clinical Experimental Thoracic Oncology, Istituto Nazionale Tumori, IRCCS, Fondazione Pascale, Naples, Italy. [3]Department of Experimental Medicine, Università degli Studi della Campania Luigi Vanvitelli Napoli, IT, Naples, Italy. [4]Institute of Cancer Research, Department of Medicine I, Comprehensive Cancer Center, Medical University of Vienna, Borschkegasse 8a, Vienna, Austria. [5]Cell Biology and Biotherapy Unit, Istituto Nazionale Tumori, IRCCS, Fondazione Pascale, Naples, Italy. [6]Medical Oncology, Hospital Casa Sollievo Della Sofferenza–San Giovanni Rotondo (Foggia), San Giovanni Rotondo, Italy. [7]Biostatistics, Dipartimento di Salute Mentale e Fisica e Medicina Preventiva, Università degli Studi della Campania L. Vanvitelli, Naples, Italy.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Authors: Erika Martinelli, Medical Oncology, Department of Precision Medicine, Università degli Studi della Campania L. Vanvitelli, Via Pansini 5, 80131 Naples, Italy. Phone: 39-081-5666628; Fax: 39-081-5666732; E-mail: erika.martinelli@unicampania.it; and Fortunato Ciardiello, E-mail: fortunato.ciardiello@unicampania.it

doi: 10.1158/1535-7163.MCT-18-0539

downstream signaling proteins, such as *KRAS*, *NRAS*, and *BRAF*, or in genes encoding for other growth factor receptor tyrosine kinases, including *HER2* and *MET*, drive primary (intrinsic) cancer cell resistance (5). Different molecular mechanisms that cause secondary (acquired) cancer cell resistance have been identified, and they partially overlap with those responsible for primary resistance (6). However, there is a need to better define these mechanisms in order to render more effective anti-EGFR therapies in metastatic colorectal cancer management (6).

Erythropoietin-producing hepatocellular (EPH) A2 receptor belongs to the EPH receptor tyrosine kinase (RTK) family classified in two subfamilies (EPHA and EPHB) according to sequence homology and binding to ligands called "ephrins" (7–8).

Ephrin-dependent signaling plays a role in cancer cell growth, migration, and invasiveness through various pathways including RAS and AKT, integrin-mediated adhesion, and epithelial-to-mesenchymal transition (EMT; refs. 7–9). In a variety of malignancies, different ephrins and/or its receptors, including EPHA2, are activated, potentially contributing to increased malignancy and poor prognosis (7–8, 10). In colorectal cancer, EPHA2 and ephrin A1 were found significantly overexpressed in stages I to II compared with stages III to IV, suggesting a potential role in the early stages of disease development (11). EPHA2 has been also associated with poor prognosis in stages II and III (11). It is also involved in cancer cell resistance to anti-EGFR tyrosine kinase inhibitors in non–small cell lung cancer (NSCLC) and to vemurafenib in BRAF-mutant melanoma (12–13).

In the present study, we have evaluated the potential role of EPHA2 in the molecular mechanisms that cause primary and acquired resistance to anti EGFR therapies, by using human colorectal cancer cell lines *in vitro* as well as *in vivo* model of immune-deficient mice bearing human colon cancer xenografts. Finally, we have studied EPHA2 expression in colorectal cancer tissue samples from patients treated with chemotherapy (FOLFIRI) and cetuximab in the CAPRI-GOIM clinical trial (14).

Materials and Methods

Drugs and chemicals

The EPHA2 inhibitor ALW-II-41-27 was purchased from MedChem Express Italy. It was dissolved in sterile DMSO at 10 mmol/L stock solution concentration and stored in aliquots at −20°C. Cetuximab, an anti-EGFR human-mouse chimeric mAb, was kindly provided by Merck Italy, Rome (see Supporting Information for more details).

Cell lines

Human LOVO, SW620, HCT15 colorectal cancer cell lines were obtained from the ATCC and authenticated by IRCCS "Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova," Italy. The human SW48 (catalog number: HTL99020), HCT116 (catalog number: HTL95025), SW480 (catalog number: HTL99017) DMSO cell lines were obtained from IRCCS "Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova," Italy. The human GEO colon cancer cell line was kindly provided by Dr. N. Normanno. GEO-CR and SW48-CR cells were established as previously described in our laboratory (15–16) (see Supporting Information for more details).

Proliferation assay

Cell proliferation was analyzed by the MTT assay. Cell suspensions (2000 μL) containing10,000 viable cells were plated in 24 multi-well plates. After 24 hours, cells were treated with different concentrations of ALW-II-41-27 (0.01, 0.05, 0.1, 0.5, 1, or 2 μmol/L) or cetuximab (0.05, 0.25, 0.5, 2.5, 5, or 10 μg/mL) as single agents or in combination for 96 hours (see Supporting Information for more details).

Protein expression analysis

Protein lysates containing equal amount of proteins, measured by a modified Bradford assay (Bio-Rad), were subjected to direct Western blot. Immunocomplexes were detected with the enhanced chemiluminescence kit (ECL plus, Thermo Fisher Scientific). We used the following antibodies from Cell Signaling Technology: anti-EGFR, antiphospho-EGFR (Tyr1068), anti-p44/42 MAPK, antiphospho-p44/42MAPK, anti-AKT, antiphospho-AKT (Ser 473), anti-EPHA2, antiphospho-EPHA2, antiphospho-S6 ribosomal protein, anti-PARP. Anti-α-tubulin (internal loading control) was from Sigma-Aldrich. The following secondary antibodies from Bio-Rad were used: goat anti-rabbit IgG and rabbit anti-mouse IgG. Each experiment was done in triplicate.

Human phosphokinase array

The phosphorylation status of a wide range of RTKs was evaluated in SW48 and SW48-CR colon cancer cell lines. To do this, a commercial array was used (Human Phospho-Kinase Array Kit; R&D Systems). According to the manufacturer's instructions, antibody array membranes were incubated overnight at 4°C with 300 μg of protein lysates. Afterward, the membranes were washed and exposed to chemiluminescent reagent to analyze the phosphorylation profile of the 49 kinases.

RNA interference

The small inhibitor duplex RNAs (siRNA; ON-target plus SMARTpool) si-Human EPHA2 (cat. #L-003116-00, Lot #130400) was from Dharmacon. The siCONTROL Nontargeting Pool (#D-001206-13-05) was used as a negative (scrambled) control. Cells were transfected with 100 nmol/L siRNAs using Dharmafect reagent following the manufacturer's instructions. The day before transfection, cells were plated in 35-mm dishes at 40% of confluence in medium supplemented with 5% FBS without antibiotics. Cells were harvested at 96 hours after transfection.

Indirect immunofluorescence

SW48-CR cells (5×10^4) were seeded on cover glass dishes and incubated with cetuximab (5 μg/mL) or ALW-II-41-27 (1 μmol/L), as single agents or in combination for 24 hours. After each treatment, cells were rinsed twice with PBS and fixed with 4% paraformaldehyde for 20 minutes. Then, cells were treated with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 minutes and with PBS-BSA 0.5% for 30 minutes at room temperature. EPHA2 expression was detected by incubating each sample with a primary rabbit anti-EPHA2 antibody (Cell Signaling Technology). Afterward, Alexa Fluor488 goat anti-rabbit secondary antibodies (Molecular Probes, Invitrogen) were used. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Samples were observed by confocal microscope system using a 63× oil immersion objective. Confocal images were acquired with a resolution of 1024 × 1024 pixels. Images for all

conditions were obtained using identical acquisition parameters, and the fluorescence intensity were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/).

Apoptosis assay and cell-cycle analysis

Cells were seeded in 6-well plates, treated for 72 hours, and stained with Annexin V–fluorescein isothiocynate (FITC). Apoptotic cell death was assessed by counting the numbers of cells that stained positive for Annexin V–FITC and negative for propidium iodide (Sigma-Aldrich) by using an Apoptosis Annexin V–FITC Kit (Invitrogen), coupled with fluorescence-activated cell sorting analysis. Cell-cycle analysis assays were performed by using flow cytometry (see Supporting Information for more details).

Tumor xenografts in nude mice

Four- to six-week-old female balb/c athymic (nu+/nu+) mice were purchased from The Charles River Laboratories. The research protocol was approved, and mice were maintained in accordance with the institutional guidelines of the Università degli Studi della Campania L. Vanvitelli Animal Care and Use Committee. Animal care was in compliance with Italian (Decree 116/92) and European Community (E.C. L358/1 18/12/86) guidelines on the use and protection of laboratory animals. Mice were acclimatized at Università degli Studi della Campania L. Vanvitelli Medical School Animal Facility for 1 week prior to being injected with cancer cells and then caged in groups of five. A total of 3×10^6 SW48 cells were resuspended in 200 μL of Matrigel (BD Biosciences) and PBS (1:1) and implanted subcutaneously into the right flank of 21 nude female mice. At week 2, once tumors reached a mean volume of 120 mm^3, mice were randomized into treatment group (11 mice) or control group (10 mice), to receive treatment with cetuximab 25 mg/kg or vehicle (10% 1-methyl-2-pyrrolidinone and 90% PEG 300), respectively, via intraperitoneal injection, 2 days a week. At week 8, 10 mice of the treatment group were assigned to receive ALW-II-41-27 (30 mg/kg) via intraperitoneal injection in combination with cetuximab. One mouse was euthanized and tumor collected for molecular analysis. Treatment with ALW-II-41-27 was performed for 4 weeks. At week 12, mice were euthanized, and tumor specimens were taken for molecular analyses. Mice body weights were monitored daily. A total of 3×10^6 HCT15 cells were resuspended in 200 μL of Matrigel (BD Biosciences) and PBS (1:1) and implanted subcutaneously into the right flank of 40 nude female mice. At week 2, once tumors reached a mean volume of 208 mm^3, mice were randomized in groups of 10 into 4 arms to receive: vehicle (10% 1-methyl-2-pyrrolidinone and 90% PEG 300) via intraperitoneal injection, 2 days a week; cetuximab 25 mg/kg via intraperitoneal injection, 2 days a week; ALW-II-41-27 (30 mg/kg) daily via intraperitoneal injection and ALW-II-41-27 in combination with cetuximab. Tumor size was evaluated twice a week by caliper measurements using the following formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. For assessment of tumor response to treatment, we used volume measurements and adapted clinical criteria (see Supporting Information for more details).

Patients

Formalin-fixed paraffin-embedded primary tumor tissues from 82 *RAS* wild-type (WT) specimens, determined by next-generation sequencing (NGS) within the CAPRI-GOIM trial (14), were assessed for EPHA2 expression by immunohistochemistry. The study, a nonprofit academic, open-label multicenter trial (EudraCT, number 2009-014041-81), enrolled 340 KRAS exon-2 WT metastatic colorectal cancer patients, according to local laboratory assessment, treated in first line with FOLFIRI and cetuximab, was approved by local ethical committees in 25 participating centers in Central and Southern Italy, and all patients gave written informed consent. The study was conducted according to GCP/ICH guidelines and the Declaration of Helsinki (Supporting Information for more details).

Immunohistochemistry

Sections 4-μm-thick were deparaffinized and rehydrated. Antigen retrieval was carried out in pH 9.0-target retrieval solution (S236784, Dako) in an antigen retrieval machine using standard protocols. Slides were washed with TBS-T, containing 0.1% Tween. Endogenous peroxidase was blocked for 10 minutes in 3% H_2O_2. Unspecific binding was blocked for 1 hour in TBS-T, 2% BSA, 5% goat serum. The primary EPHA2 antibody was incubated overnight at 4°C at the concentration of 1:200 (#6997, Rabbit mAb, Cell Signaling Technology) in blocking solution. Slides were washed and then incubated with the Signal Stain Boost IHC Detection Reagent (#8114, Cell Signaling Technology). DAB reaction was carried out for 10 minutes using the DAB Substrate Kit (SK-4100, Vector; see Supporting Information for more details). Intensity was assessed according to the following scores: 0 = negative; 1 = weak; 2 = moderate; 3 = intense. Histoscore (HSCORE) was calculated by a semi-quantitative assessment of both the intensity of staining and the percentage of positive cells [1 × (% cells weak) + 2 × (% cells moderate) + 3 × (% cells intense)], obtaining a final score ranging from 0 to 300. Receiver operator characteristic analysis was used to set a cutoff to define high (HSCORE >50) and low (HSCORE ≤50) EPHA2 levels (see Supporting Information for more details). Evaluation was performed by two experts (M.C. Paul and V. Moreno-Viedma), without any prior knowledge of clinicopathologic information.

Statistical analysis

Differences between categorical data were measured by χ^2 and Fisher exact test when appropriate. Differences between continuous variables were investigated by the Mann–Whitney *U* test and Kruskal–Wallis test, when appropriate. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. All analyses were two-sided, with *P* value <0.05 being considered to indicate statistical significance. Analyses were performed using the SPSS package (version 22.0 for Windows, SPSS Inc.).

Results

Expression and activation of EPHA2 in human colorectal cancer cell lines

We analyzed the expression and activation of EPHA2 in a panel of colorectal cancer cell lines with both intrinsic (HCT116, SW620, LOVO, SW480, and HCT15) or acquired resistance (GEO-CR and SW48-CR cells) to the anti-EGFR mAb cetuximab, as well as with sensitivity to cetuximab (GEO and SW48). The highest levels of EPHA2 phosphorylation were detected in LOVO, HCT116, SW620, HCT15, and SW48-CR cells (Fig. 1A), by Western blot analysis.

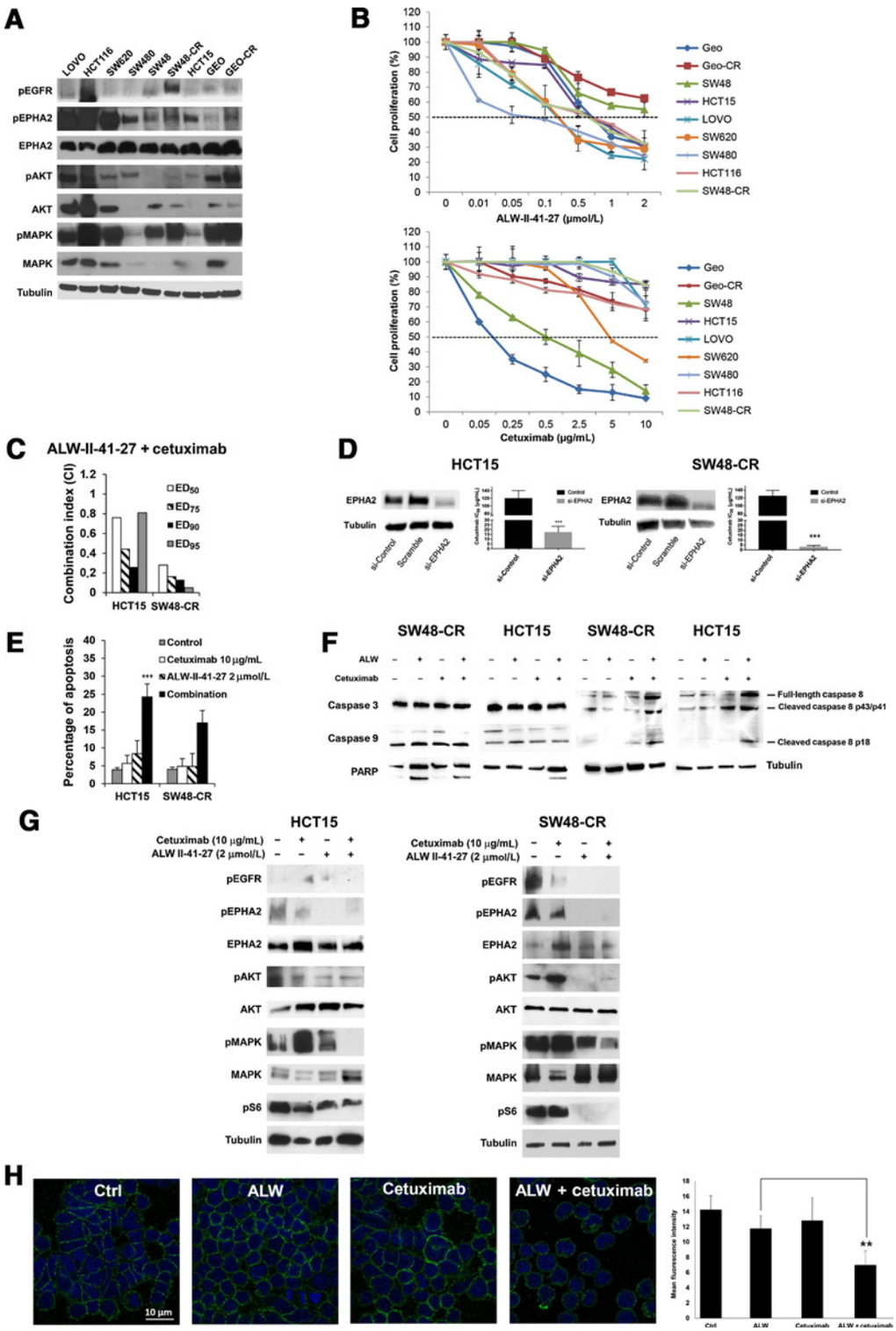


Figure 1.

The *in vitro* effect of EPHA2 inhibition on a panel of human colorectal cancer. **A,** Basal EPHA2 expression and activation in a panel of human colorectal cancer cell lines. WB analysis defined the expression and activation of EPHA2 in LOVO, HCT116, SW620, HCT15, SW480, GEO, GEO-CR, SW48, and SW48-CR human colorectal cancer cell lines. Thirty micrograms of cell protein extracts was subjected to immunoblotting and incubated with the appropriate antibodies as described in Materials and Methods. Anti-tubulin antibody was used for normalization of protein extract content. (*Continued on the following page.*)

***In vitro* effects of treatment with the specific EPHA2 inhibitor, ALW-II-41-27, on human colorectal cancer cell growth**

To determine the effects of EPHA2 inhibition, we used a specific inhibitor, ALW-II-41-27, a type II small-molecule inhibitor that targets the ATP-binding pocket of the kinase domain as well as an allosteric site next to the "DFG" motif in the EPHA2 (13, 17). The human colorectal cancer cell lines tested were differently sensitive to ALW-II-41-27 antiproliferative effect as single agent, with an IC_{50} ranging from 0.05 to >2 μmol/L (Fig. 1B; Supplementary Table S1). All human colorectal cancer cell lines with *KRAS* mutation were resistant to cetuximab (IC_{50} >10 μg/mL), with the exception of GEO (as previously shown; refs. 15 and 16) and SW48 cells, the latter known to be *RAS* WT (Fig. 1B; Supplementary Table S1). In the absence of a correlation with ALW-II-41-27 activity and p-EPHA2 levels, we have performed a phospho-RTK array with cetuximab-sensitive (SW48) versus -resistant (SW48-CR) cell lines in order to assess the spectrum of RTK activation. We have selected these cell lines as they retain a different sensitivity also for ALW-II-41-27, being SW48 considered resistant (IC_{50} >2 μmol/L) and SW48-CR sensitive (IC_{50} 0.75 μmol/L). We found in cetuximab-resistant cells an activation of EPHA2 compared with the sensitive ones. No difference in other putative ALW-II-41-27 targets was seen in sensitive versus resistant cells. In fact, the experiment shows an increase in phosphorylation of EPHA10, EPHA1, EPHA6, HER2, HGFR, IGF1, ROR, and ALK in SW48-CR cells compared with SW48, respectively. All these kinases are not considered principal targets of ALW-II-41-27 (Supplementary Fig. S1A).

Moreover, we also tested the effect of ALW-II-41-27 on EPHA2 siRNA in HCT15 and SW48-CR cells. After the transient knockdown, these two cell lines became refractory to ALW-II-41-27, suggesting that the effect we were observing was mediated by EPHA2 indeed (Supplementary Fig. S1B).

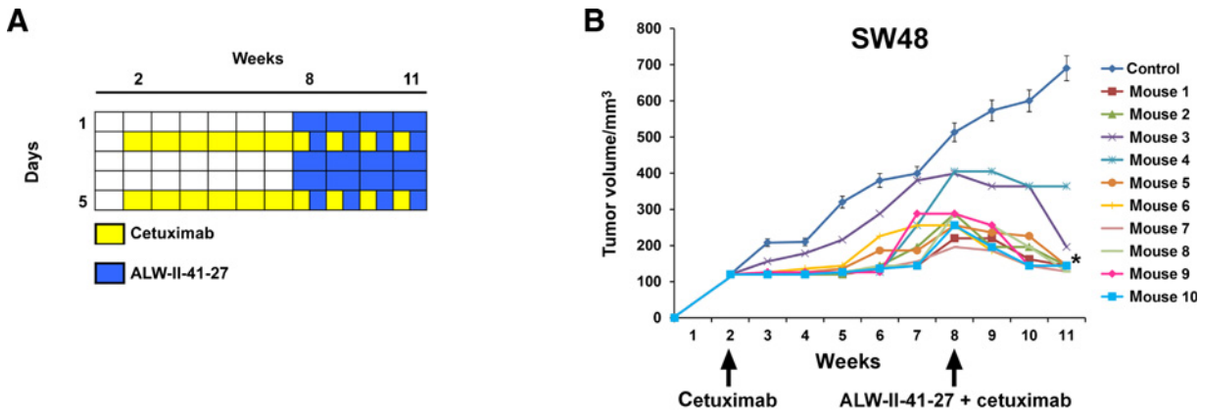
EPHA2 inhibition restores sensitivity to cetuximab in human colorectal cancer cell lines

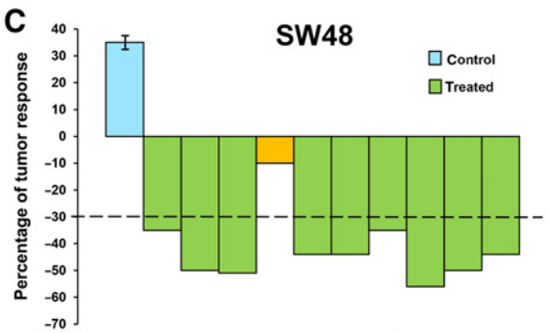
To evaluate whether EPHA2 functional inhibition could contribute to revert resistance to anti-EGFR therapy, the human colorectal cancer cell lines were treated with a combination of different concentrations of ALW-II-41-27 (range, 0.01–2 μmol/L) and cetuximab (range, 0.05–10 μg/mL) for a total of 4 days at a fixed 1:5 drug ratio (ALW-II-41-27: cetuximab); by combination index (CI) analysis, a synergistic, antiproliferative effect was found on cells with both intrinsic (HCT15) and/or acquired (SW48-CR and GEO-CR) resistance to cetuximab and in GEO cells, with a CI lower than 1 at ED50 (values from 0.28 to 0.89; Fig. 1C; Supplementary Fig. S2A). The result was different in other human colorectal cancer cell lines analyzed, with intrinsic resistance to cetuximab such as HCT116, SW620, LOVO, and SW480, in which the CI was higher than 1 and, therefore, the effect resulted antagonistic (Supplementary Fig. S2B). Moreover, following *EphA2* gene-expression inhibition, by using a small interference RNA approach, cetuximab sensitivity was restored in both HCT15 and SW48-CR cell lines (Fig. 1D). On the other hand, silencing of *EphA2* in two cell lines where the combination was antagonistic, respectively, HCT116 and LOVO, did not induce cetuximab sensitivity (Supplementary Fig. S2C).

Effect of the EPHA2 inhibitor in combination with cetuximab on intracellular signaling, induction of apoptosis and cell-cycle analysis in human colorectal cancer cell lines

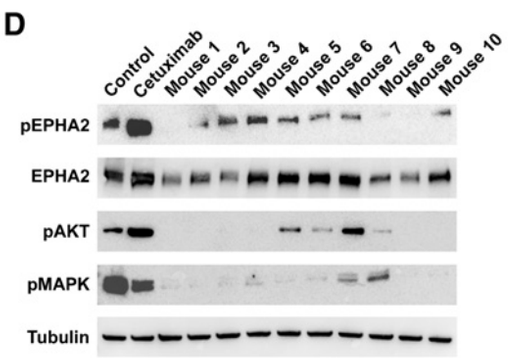
Next, we investigated the impact of the combined treatment on apoptosis and cell-cycle distribution. We evaluated the ability of ALW-II-41-27 alone or in combination with cetuximab to induce apoptosis by using Annexin V–FITC assay. The combination of the two drugs, as compared with single-agent treatments, was able to determine a significant increase in apoptosis only in HCT15, SW48-CR, GEO, and GEO-CR, the four human colorectal cancer cell lines in which the antiproliferative effects of the two drugs resulted synergistic (Fig. 1E; Supplementary Fig. S2D). Western blot analysis of HCT15 and SW48-CR cell lines confirmed the increase in apoptosis related to drug combination (Fig. 1F). Because in SW48-CR cell lines we did not find any difference with the combination treatment in cleaved PARP, compared with ALW-II-41-27 alone, we analyzed the caspase 8, that is known to initiate apoptotic signaling via the extrinsic pathway, finding an increased cleavage after the combination treatment, compared with single-agent ALW-II-41-27 (Fig. 1F).

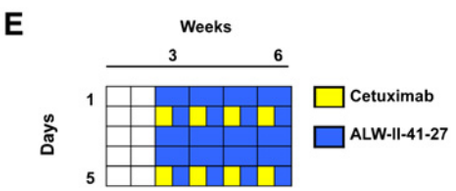
(*Continued.*) **B,** Treatment with ALW-II-41-27 or cetuximab as single agents. Human colorectal cancer cell lines were treated with ALW-II-41-27 (drug concentrations range from 0.01 to 2 μmol/L) or cetuximab (drug concentrations range from 0.05 to 10 μg/mL) for 96 hours. Cell viability was assessed by MTT assay. The results are average ± SD of 3 independent experiments each done in quadruplicate. **C,** Synergistic antiproliferative effects of the combination of ALW-II-41-27 and cetuximab. The indicated colorectal cancer cell lines were treated with ALW-II-41-27 (range, 0.01–2 μmol/L) and cetuximab (range, 0.05–10 μg/mL) for a total of 4 days at a fixed 1:5 drug ratio (ALW-II-41-27: cetuximab); proliferation was evaluated by MTT. Combination index (CI) analysis with Calcusyn (Biosoft) program was used to determine the synergistic effect of ALW-II-41-27 and cetuximab. ED_{50}, ED_{75}, ED_{90}, and ED_{95} concentrations were defined. In particular, combination of the two drugs determined a synergistic effect, CI < 1. **D,** EPHA2 mediates resistance to cetuximab. The knockdown of *Epha2* by using a small inhibitor si-Human EPHA2 was evaluated in HCT15 and SW48-CR cell lines. Cells were harvested at 96 hours after transfection. Western blot for EPHA2 expression was performed. IC_{50} for cetuximab was calculated in control and transfected cells. All bars indicate IC_{50} mean value ± SD. Statistically significant differences were calculated using Student *t* test: ***, $P < 0.0001$. **E–F,** Effects of combination treatment with ALW-II-41-27 and cetuximab on induction of apoptosis: Apoptosis was evaluated with Annexin V staining, as described in Materials and Methods. Cancer cells were treated for 72 hours with cetuximab, ALW-II-41-27 alone or their combination. The rate of apoptosis was expressed as a percentage of the total cells counted. Columns are the means of 3 independent experiments; statistically significant differences were calculated using Student *t* test: ***, $P < 0.0001$. Expression of cleaved form of PARP for HCT15 and SW48-CR was evaluated by immunoblotting. Anti-tubulin antibody was used for normalization of protein extract content. **G,** Effects of EPHA2 blockade alone and in combination with cetuximab on intracellular signaling pathways of cell proliferation and survival. The effects of ALW-II-41-27 as single agent and/or in combination with cetuximab were analyzed by Western blot in HCT15 and SW48-CR cells. Thirty micrograms of cell protein extracts was fractioned through 4%–20% SDS-PAGE, transferred to nitrocellulose filters and incubated with the appropriate antibodies as described in Materials and Methods. **H,** Indirect immunofluorescence. SW48-CR cells (5×10^4) were seeded on cover glass dishes and incubated with cetuximab (5 μg/mL) and ALW-II-41-27 (1 μmol/L), as a single agent or in combination for 24 hours as described in Materials and Methods. Samples were observed by confocal microscope using a 63× oil immersion objective. Images were acquired with a 1024 × 1024 resolution. EPHA2 was stained with a primary anti-EPHA2 rabbit antibody coupled with an FITC-labeled secondary antibody. Nuclei are visualized by DAPI staining. The fluorescence intensity was quantified using ImageJ software. Statistical significance was determined using ANOVA; **, $P < 0.01$. Scale bar, 10 μm.

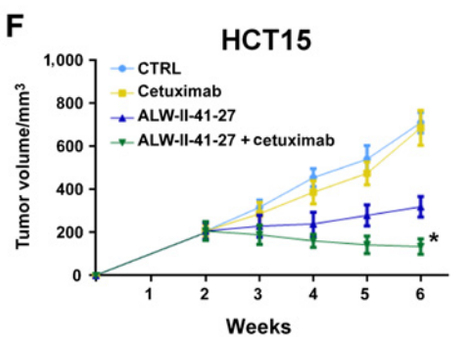




Combination	Volume mm³ week 8	Volume mm³ week 11	Best response
Control	513	690	PD
Mouse 1	220	144	PR
Mouse 2	288	144	PR
Mouse 3	399	196	PR
Mouse 4	405	364	SD
Mouse 5	256	144	PR
Mouse 6	256	144	PR
Mouse 7	196	128	PR
Mouse 8	288	128	PR
Mouse 9	288	144	PR
Mouse 10	256	144	PR







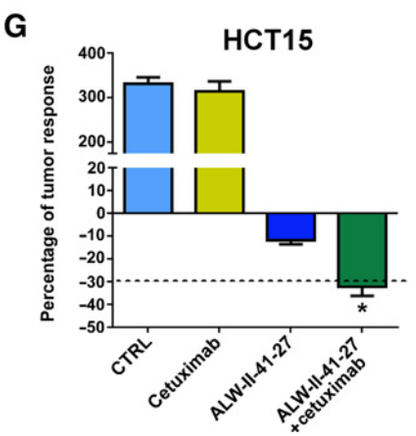


Figure 2.

The *in vitro* effect of EPHA2 inhibition on a panel human of colorectal cancer. **A–D**, Antitumor activity of ALW-II-41-27 plus cetuximab in SW48 and HCT15 (**E–G**) xenograft models. **A** and **B**, Treatment schedule and effects of ALW-II-41-27 plus cetuximab on tumor growth. SW48 human colorectal cancer cells were injected subcutaneously into the right flank of 21 nude mice. After 2 weeks, once tumors reached a volume of 120 mm^3, mice were randomized to control group (10 mice) to receive vehicle alone (10% 1-methyl-2-pyrrolidinone and 90% PEG 300) and to treatment group with cetuximab (25 mg/kg) intraperitoneally, 2 days a week (11 mice). Treatment was continued until disease progression. At progression (week 8), when the tumors were considered resistant to cetuximab, one mouse was taken for molecular analysis of the tumor sample, and 10 mice were treated with ALW-II-41-27 (30 mg/kg) daily plus cetuximab, intraperitoneally, 2 days a week. Treatment was conducted for 4 weeks (week 11), then mice were euthanized, and molecular analysis of tumor samples was carried out. Black arrows indicate the time of starting treatment (week 2) and time of progression to cetuximab (week 8); *, $P < 0.05$. (*Continued on the following page.*)

Treatment with ALW-II-41-27 in combination with cetuximab determined a significant and time-dependent cell-cycle arrest in G_1 in HCT15 cells and in G_2 in SW48-CR cells, as compared with single-agent treatments, with a consistent S-phase shrinkage in both cell lines (Supplementary Fig. S2E).

A strong reduction in phosphorylation of EPHA2 was found following treatment with both ALW-II-41-27 as single agent and in combination with cetuximab. This effect was paralleled by a sustained reduction of phospho-MAPK, phospho-AKT, and its downstream phospho-S6 ribosomal protein in both cell lines with acquired resistance to cetuximab (SW48-CR and GEO-CR; Fig. 1G; Supplementary Fig. S2F). Furthermore, in HCT15 cells, that possess intrinsic resistance to anti-EGFR drugs, the effect of the combined treatment was particularly significant on the MAPK pathway, with a total suppression of phospho-MAPK (Fig. 1G). On the contrary, in human colorectal cancer cell lines in which the combination had an antagonistic effect (HCT116, LOVO, and SW480), treatment with ALW-II-41-27 or with the combination was not effective in reducing MAPK phosphorylation (Supplementary Fig. S2F).

Finally, evaluation of pEPHA2 abundance on SW48-CR cells by indirect immuno-fluorescence showed significant reduction of p-EPHA2 on the cancer cell membrane by treatment with ALW-II-41-27, which was even more pronounced following the combined treatment with cetuximab (Fig. 1H).

Antitumor activity of ALW-II-41-27 in combination with cetuximab on human SW48 and HCT15 tumor xenografts resistant to cetuximab

We next investigated the potential role of treatment with the EPHA2 specific inhibitor ALW-II-41-27 in restoring antitumor activity of cetuximab in *in vivo* models of intrinsic and acquired resistance. SW48 cells, known to be very sensitive to EGFR inhibition, were subcutaneously injected into the right flank of 21 mice. Once tumors reached a mean volume of 120 mm^3, mice were randomized into the treatment group (11 mice) to receive cetuximab (25 mg/kg), or into the control group (10 mice) to receive vehicle alone (10% 1-methyl-2-pyrrolidinone and 90% PEG 300), via intraperitoneal injection, 2 days a week (Fig. 2A). On week 8, all 11 mice became resistant to cetuximab, as an increase in tumor volume more than 20% was reported; thus, a daily treatment with ALW-II-41-27 (30 mg/kg) via intraperitoneal injection in combination with cetuximab twice a week was started in 10 mice. One mouse was euthanized, and its tumor was collected for further molecular analyses. In the combination group, after 4 weeks of treatment, tumor volumes resulted significantly reduced as compared with control and with tumor volumes at the beginning of the treatment (Fig. 2B). In particular, 9 of 10 mice obtained a partial response (PR) with a tumor reduction greater than 35% (Fig. 2C). Treatment was well tolerated with no sign of acute or delayed toxicity. WB analysis of tumor specimens found that the combined treatment with ALW-II-41-27 and cetuximab caused significant reduction in EPHA2, MAPK, and AKT phosphorylation levels (Fig. 2D). As a further experiment, HCT15 human colorectal cancer cell lines were subcutaneously inoculated in the right flank of 40 nude mice. Once tumors reached a mean volume of 208 mm^3, mice were randomized into 4 groups of 10 to receive vehicle, cetuximab alone, ALW-II-41-27 alone, and a combination of ALW-II-41-27 with cetuximab, as described in Materials and Methods (Fig. 2E and F). After 4 weeks of treatment, mice randomized in the combination arm achieved a median PR of 35%, compared with the ALW-II-41-27 arm in which mice experienced a stability of disease (response less than 30%) and with cetuximab and control arms in which progression of disease occurred in all mice (Fig. 2G).

EPHA2 expression in metastatic colorectal cancer patients from the CAPRI-GOIM trial

We have previously reported that in all *RAS* WT patients, as assessed by NGS (5) and by liquid biopsy in the CAPRI-GOIM clinical study (14), FOLFIRI plus cetuximab was an effective first-line treatment, with median PFS 13.8 months (CI 95%, 10.5–17.0) and median OS 35.8 months (CI 95%, 31.0–40.6). Eighty-two *RAS* WT tumor specimens from patients with metastatic colorectal cancer, treated with FOLFIRI plus cetuximab, as first-line therapy, and representative of the entire 124 *RAS* WT NGS-assessed patient population which was enrolled in the CAPRI-GOIM trial (18, 19), were assessed for EPHA2 expression by immunohistochemistry. Fifty-five of 82 (67%) cases were positive for EPHA2 expression. Positivity resulted in mostly complete membranous staining in cancer cells. According to the intensity score staining (see Materials and Methods), EPHA2 expression was intense in 1 case, moderate in 10 cases, and weak in 44 cases (Fig. 3A; Supplementary Table S2). Tumor stroma stained positively in 15 of 82 (18%) specimens (Supplementary Table S2). In most of these cases, an intense immune infiltrate, mainly characterized by tumor-associated macrophages, was observed. Nontumor adjacent normal mucosa was assessable in 34 of 82 samples. EPHA2 was expressed in 16 of 34 samples (47%), more frequently in dysplastic epithelial areas (Supplementary Table S2).

To better evaluate EPHA2 expression, in order to take in account intratumor heterogeneity, a semiquantitative immunehistoscore (HSCORE) method was developed (as described in Materials and Methods). Using a threshold based on HSCORE

(*Continued.*) **C,** Best antitumor response in colorectal cancer xenograft after the end of treatment. Waterfall plot of response to ALW-II-41-27 plus cetuximab normalized against tumor volume at baseline compared with vehicle alone. The control bar represents the mean tumor volume ± SD; the other bars represent the change of tumor volume of individual mice. Cases experiencing PR and disease stabilization are shaded in light green and orange, respectively. **D,** Effects of combination of ALW-II-41-27 plus cetuximab on intracellular signaling pathways. Control and cetuximab indicate, respectively, a sample from mice treated with vehicle alone and one from the mouse treated with cetuximab and then euthanized at progression of disease. Tumor samples were collected, and total cell protein extracts were subjected to immunoblotting, as described in Materials and Methods. **E,** Treatment schedule. HCT15 human colorectal cancer cells were injected subcutaneously into the right flank of 40 nude mice. After 2 weeks, once tumors reached a volume of 208 mm^3, mice were randomized in groups of 10 into four arms to receive the following: vehicle alone, cetuximab, ALW-II-41-27, or their combination (schedule, dosing, and route of administration are the same as previously described). **F,** Effects of ALW-II-41-27, cetuximab, or their combination on tumor growth; *, $P < 0.05$. **G,** Best antitumor response in colorectal cancer xenograft after the end of treatment. Waterfall plot of response to cetuximab, ALW-II-41-27, or their combination normalized against tumor volume at baseline. The bars represent mean tumor volume ± SD; *, $P < 0.05$.

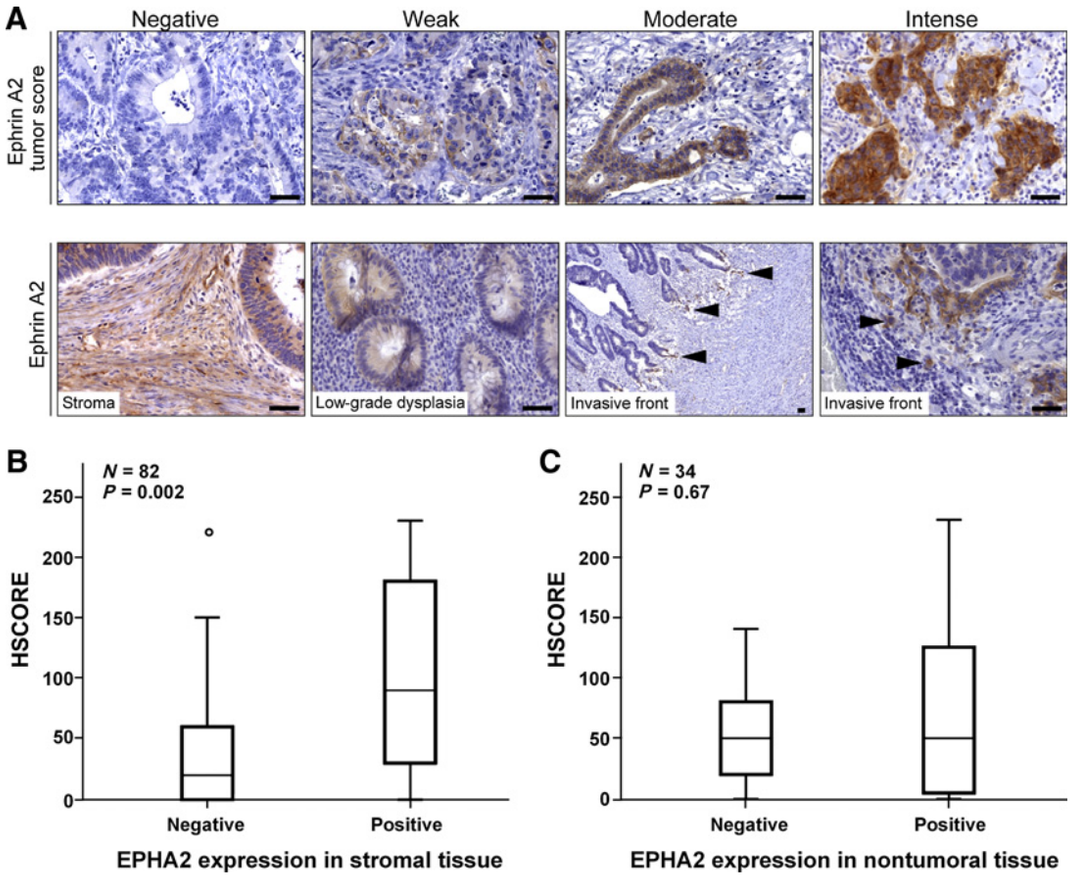


Figure 3.

EPHA2 expression in tumor samples of 82 *RAS* WT colorectal cancer patients from the GOIM-CAPRI trial. **A,** EPHA2 intensity score in tumor tissue (negative, weak, moderate, and intense) and nontumoral tissues. EPHA2 expression (HSCORE) in tumor tissue was correlated to its expression within the stromal tissue (**B**) and the nontumoral associated mucosa (**C**) by using nonparametric Mann–Whitney *U* test ($P = 0.002$ and $P = 0.77$, respectively).

(see Materials and Methods), tumor samples were classified as EPHA2 high expressers (HSCORE >50; 28 cases, 34%) and EPHA2 low expressers (HSCORE ≤50; 54 cases, 66%; Supplementary Table S3). Interestingly, EPHA2 expression in stromal tissues was significantly associated with higher HSCORE levels ($P = 0.002$; Fig. 3B). No significant correlation between EPHA2 levels in nontumor adjacent normal mucosa and in tumor tissues was observed (Fig. 3C).

Correlation between EPHA2 HSCORE and treatment efficacy in metastatic colorectal cancer patients from the CAPRI-GOIM trial

Patients' characteristics are summarized in Table 1. The median follow-up was 69 months. No significant correlation in terms of age, metastatic disease at diagnosis, patient gender, tumor size, and nodal involved was found. Interestingly, high levels of EPHA2 expression were found in less-differentiated tumors ($P = 0.02$) as well as in left-sided as compared with right-sided tumors ($P = 0.04$). Although no significant difference in the two groups was observed in overall RR to the first-line treatment with FOLFIRI plus cetuximab, a significant increase in the rate of progression was found in patients with high EPHA2 HSCORE, as compared with patients with low EPHA2 HSCORE (29% and 9%, respectively; $P = 0.02$; Table 2). Furthermore, median PFS was significantly shorter in patients with high EPHA2 HSCORE (8.6 months; 95% CI, 6.4–10.8) as compared with patients with low EPHA2 HSCORE (12.3 months; 95% CI, 10.4–14.2; $P = 0.03$; Fig. 4A). Of note, one patient whose tumor was negative for EPHA2 expression was still experiencing a complete response to treatment with FOLFIRI plus cetuximab at the time of data analysis, with a PFS of 72.2 months. Similarly, a trend to a reduced median OS was observed in patients with high EPHA2 HSCORE as compared with patients with low EPHA2 HSCORE (28.4 months; 95% CI, 13.1–43.7 and 39.8 months; 95% CI, 30.2–49.4, respectively), although this result did not reach statistical significance ($P = 0.23$; Fig. 4B).

Discussion

EPHA2 is generally overexpressed in different human malignancies, including colorectal cancer, in which it has previously been suggested as an independent adverse prognostic marker in early-stage disease (7–8, 11). Recent findings have supported EPHA2's role in cancer resistance to different targeted therapies in melanoma (13), in NSCLC (12), and in breast cancer (20).

Table 1. EPHA2 expression and correlation with clinical features in 82 *RAS* WT colorectal cancer patients from the GOIM-CAPRI trial

		EPHA2 expression (HSCORE)		
Clinical features ($N = 82$)		**Low ≤50 $N = 54$ (%)**	**High >50 $N = 28$ (%)**	***P* value**
T	1	1 (2)	0 (0)	0.45
	2	2 (4)	3 (13)	
	3	38 (72)	15 (65)	
	4	12 (23)	5 (22)	
	N/A	1 (–)	5 (–)	
N	0	13 (25)	6 (26)	0.92
	1	19 (36)	9 (39)	
	2	21 (40)	8 (35)	
	N/A	1 (–)	5 (–)	
M	0	10 (19)	3 (11)	0.53
	1	44 (81)	25 (89)	
Tumor location	Right	10 (18)	11 (39)	0.04
	Left	44 (82)	17 (61)	
Grading	1	5 (10)	1 (4)	0.02
	2	43 (83)	17 (65)	
	3	4 (8)	8 (31)	
	N/A	2 (–)	2 (–)	
Sex	M	19 (35)	11 (39)	0.81
	F	35 (65)	17 (61)	
Age at diagnosis		61.5 (28.6–77.1)	59.9 (29.9–80.5)	0.43

NOTE: EPHA2 expression (HSCORE) and correlation with patients' clinical features.

However, the contribution of EPHA2 activation and/or overexpression in metastatic colorectal cancer to therapeutic resistance is not yet fully understood.

In the present study, we have identified EPHA2 overexpression and activation as a novel mechanism of cancer resistance to treatment with anti-EGFR antibodies, such as cetuximab. In this respect, here we report that EPHA2 is overexpressed in a panel of different human colorectal cancer cell lines with primary resistance to cetuximab. Of note, in two human colorectal cancer cell lines (GEO-CR and SW48-CR) with acquired resistance to cetuximab, that have been obtained in our laboratory, increased levels of phospho-EPHA2 were detected, suggesting that EPHA2 may play a key role in inducing cetuximab resistance. Furthermore, pharmacologic inhibition of EPHA2 activation and downstream signaling by treatment with the small-molecule tyrosine kinase inhibitor ALW-II-41-27 determined cell growth inhibition and induction of apoptosis in a dose-dependent fashion in human colorectal cancer cell lines that were resistant to cetuximab. Moreover, in both cells with intrinsic (HCT15) and acquired resistance to cetuximab (GEO-CR and SW48-CR), the combined treatment with ALW-II-41-27 and cetuximab restored the efficacy of the anti-EGFR with synergistic antiproliferative and proapoptotic effects and with G_1–G_2 cell-cycle phase arrest. These results were confirmed by specific inhibition of *EphA2* gene expression, which was able to significantly increase cetuximab sensitivity in HCT15 and SW48-CR cells. These results are in agreement with the hypothesis for a key role of EPHA2 in cancer resistance to anti-EGFR blockade (20). Moreover, the combination of the anti-EPHA2 ALW-II-41-27 plus the anti-EGFR cetuximab significantly inhibited both AKT- and MAPK-activated intracellular signaling, suggesting that activation of AKT and MAPK signals is a major mechanism by which EPHA2 contributes to cetuximab cancer cell resistance. These data are consistent with previous findings and support the presence of a functional interaction between EPHA2 and the RAS pathway in cancer (11, 21). In this respect, it has been shown that EPHA2 activates the ERK1/2 pathway in human breast and prostate cancer cells (22). Similarly, in our model, the combination of cetuximab and ALW-II-41-27 is able to effectively block MAPK activation only in cell lines in which these two drugs are synergistic, suggesting that EPHA2 activation sustains MAPK phosphorylation.

Table 2. EPHA2 expression and correlation with responses to treatment

	EPHA2 expression (HSCORE)		
Objective response ($N = 82$)	**Low ($N = 54$) *N* (%)**	**High ($N = 28$) *N* (%)**	***P* value**
CR	5 (9)	1 (4)	0.35
PR	25 (46)	12 (43)	0.76
SD	19 (35)	7 (25)	0.35
PD	5 (9)	8 (29)	0.02
ORR	55%	46%	0.29

NOTE: Objective response to FOLFIRI plus cetuximab as first-line chemotherapy according to EPHA2 expression levels.

Abbreviations: CR, complete response; ORR, objective response rate; PD, progressive disease; PR, partial response; SD, stable disease.

ALW-II-41-27 treatment was also effective in overcoming both intrinsic resistance (HCT15) and acquired resistance (SW48-CR) to cetuximab in *in vivo* xenograft models. A significant reduction of tumor volumes was observed in the majority of mice and was accompanied by suppression of EPHA2, MAPK, and AKT phosphorylation, further supporting the role of EPHA2 in driving survival and proliferation in tumors with resistance to cetuximab.

The impact of EPHA2 expression on the activity and efficacy of therapy with the anti-EGFR mAb cetuximab was evaluated in a cohort of 82 *RAS* WT patients, who were enrolled in the CAPRI-GOIM trial, whose tumors were molecularly selected by NGS, and who were treated in first line with FOLFIRI plus cetuximab (14, 18–19). EPHA2 expression was found in 67% cases, with a heterogeneous pattern of staining intensity. Interestingly, EPHA2 was markedly overexpressed at the invasive tumor front, even in low EPHA2 expression specimens, providing evidence for the EPHA2 role in tumor invasion and metastasis (8). Moreover, we found EPHA2 expression both in the stroma and in the tumor-associated mucosa, in line with EPHA2 involvement in tumor microenvironment signaling. In fact, a significant correlation between higher EPHA2 levels in cancer cells and in the surrounding stroma was found, suggesting an EPHA2-activated cross-talk between tumor and host.

More importantly, within the limitation of a retrospective analysis, high levels of EPHA2 expression were predictive of significantly reduced efficacy of FOLFIRI plus cetuximab treatment in *RAS* WT metastatic colorectal cancer patients in terms of PFS and early progression. A similar observation, although not statistically significant, was reported for OS. These data further extend previous clinical reports. In fact, enhanced EPHA2 protein levels have been reported as a poor prognostic factor in early-stage, operable colorectal cancer (11); moreover, a previous study has suggested a negative predictive effect of EPHA2 expression and anti-EGFR therapy in 70 metastatic colorectal cancer patients (of which only 43 patients had a *KRAS* exon 2 WT tumor), that had a chemorefractory disease and were treated with cetuximab monotherapy as a subsequent line of therapy (23). Therefore, the present study provides the first evidence from a controlled clinical trial in all *RAS* WT metastatic colorectal cancer, treated in first line with FOLFIRI

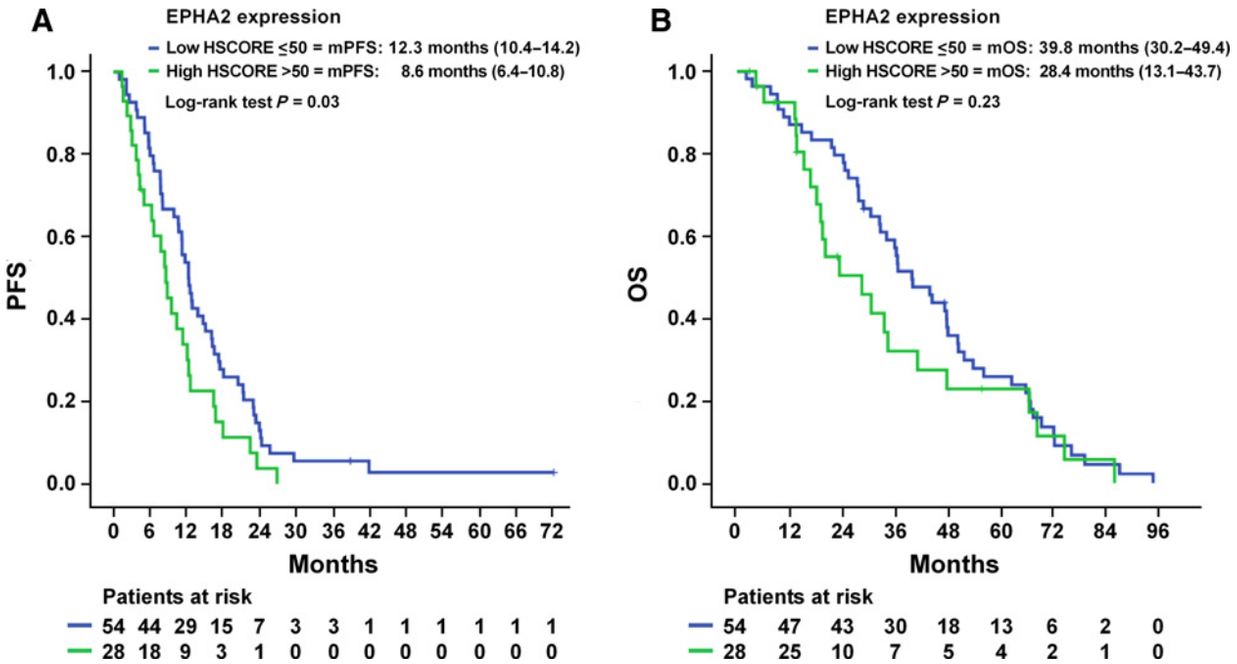


Figure 4.

EPHA2 expression and correlation with efficacy outcomes in 82 *RAS* WT colorectal cancer patients from the GOIM-CAPRI trial. PFS (**A**) and OS (**B**) according to EPHA2 expression levels.

plus cetuximab, of a role for EPHA2 overexpression in driving resistance to anti-EGFR therapies.

In summary, we have identified EPHA2 enhanced expression and activation as a mechanism of primary and acquired resistance to cetuximab and we have provided the evidence that a specific inhibitor of EPHA2 could overcome cetuximab resistance in human colorectal cancer models both *in vitro* and *in vivo*. In this respect, the complex molecular heterogeneity of metastatic colorectal cancer has not yet been completely understood and, to date, there is a lack of more precise and effective predictive biomarkers useful to select subsets of patients, whose tumors are potentially sensitive to targeted drugs. Only *KRAS* and *NRAS* mutation status is currently used as a negative predictive marker for resistance to anti-EGFR therapy in patients with metastatic colorectal cancer (24). In this setting, evaluation of EPHA2 overexpression might represent an additional predictive biomarker of lack of efficacy in *RAS* WT metastatic colorectal cancer patients. In this respect, combined anti-EPHA2 and anti-EGFR therapies could be developed for these patients. Taken together, these findings have a clinical relevance because they provide the rational basis for innovative therapeutic strategies to render more effective anti-EGFR therapies in metastatic colorectal cancer patients.

Disclosure of Potential Conflicts of Interest

N. Normanno reports receiving a commercial research grant from Merck Serono and has received honoraria from speakers bureau of Merck Serono. M. Sibilia reports receiving commercial research grant from Boehringer Ingelheim and has received honoraria from speakers bureau of the same. F. Ciardiello is a consultant/advisory board member for Roche, Merck KgA, Amgen, Servier, Bayer, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: G. Martini, C. Cardone, F. Ciardiello, E. Martinelli
Development of methodology: G. Martini, C. Cardone, V. Belli, T. Troiani, C.M. Della Corte, F. Morgillo, N. Matrone, V. Moreno-Viedma, F. Ciardiello, E. Martinelli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Martini, C. Cardone, P.P. Vitiello, V. Belli, D. Ciardiello, G. Papaccio, V. Desiderio, M.C. Paul, V. Moreno-Viedma, N. Normanno, V. Tirino, E. Maiello, D. Rizzi, F. Ciardiello, E. Martinelli
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Martini, C. Cardone, P.P. Vitiello, V. Belli, T. Troiani, D. Ciardiello, A.M. Rachiglio, G. Signoriello, M. Sibilia, E. Martinelli
Writing, review, and/or revision of the manuscript: G. Martini, C. Cardone, P.P. Vitiello, V. Belli, S. Napolitano, D. Ciardiello, C.M. Della Corte, V. Sforza, N. Normanno, F. Ciardiello, E. Martinelli
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Martini, C. Cardone, S. Napolitano, T. Troiani, C.M. Della Corte, D. Rizzi, F. Ciardiello, E. Martinelli
Study supervision: T.P. Latiano, M. Sibilia, F. Ciardiello, E. Martinelli

Acknowledgments

The authors wish to acknowledge AIRC for funding. E. Martinelli received an AIRC grant (Associazione Italiana per la Ricerca sul cancro) MFAG-2015-ID:7778; F. Ciardiello received an AIRC grant (Associazione Italiana per la Ricerca sul cancro): IG-2013-ID:14800.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 14, 2018; revised October 28, 2018; accepted February 21, 2019; published first March 1, 2019.

References

1. Van Cutsem E, Cervantes A, Adam R, Sobrero A, Van Krieken JH, Aderka D, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol 2016;27:1386–422.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87–108.
3. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359–86.
4. Martinelli E, Troiani T, Morgillo F, Orditura M, De Vita F, Belli G, et al. Emerging VEGF-receptor inhibitors for colorectal cancer. Expert Opin Emerg Drugs 2013;18:25–37.
5. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med 2008;358:1160–74.
6. Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J Clin Oncol 2010;28:1254–61.
7. Boyd AW, Bartlett PF, Lackmann M. Therapeutic targeting of EPH receptors and their ligands. Nat Rev Drug Discov 2014;13:39–62.
8. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer 2010;10:165–80.
9. Lisabeth EM, Falivelli G, Pasquale EB. Eph receptor signaling and ephrins. Cold Spring Harb Perspect Biol 2013;5:a009159.
10. Rudno-Rudzińska J, Kielan W, Frejlich W, Kotulski K, Hap W, Kurnol K, et al. A review on Eph/ephrin, angiogenesis and lymphangiogenesis in gastric, colorectal and pancreatic cancers. Chin J Cancer Res 2017;29:303–12.
11. Dunne PD, Dasgupta S, Blayney JK, McArt DG, Redmond KL, Weir JA, et al. EphA2 expression is a key driver of migration and invasion and a poor prognostic marker in colorectal cancer. Clin Cancer Res 2016;22:230–42.
12. Amato KR, Wang S, Tan L, Hastings AK, Song W, Lovly CM, et al. EPHA2 blockade overcomes acquired resistance to EGFR kinase inhibitors in lung cancer. Cancer Res 2016;76:305–18.
13. Miao B, Ji Z, Tan L, Taylor M, Zhang J, Choi HG, et al. EphA2 is a mediator of vemurafenib resistance and a novel therapeutic target in melanoma. Cancer Discov 2015;5:274–87.
14. Ciardiello F, Normanno N, Maiello E, Martinelli E, Troiani T, Pisconti S, et al. Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by next-generation sequencing: findings from the CAPRI-GOIM trial. Ann Oncol 2014;25:1756–61.
15. Troiani T, Martinelli E, Napolitano S, Vitagliano D, Giuffreda LP, Costantino S, et al. Increased TGF-a as a mechanism of acquired resistance to the anti-EGFR inhibitor cetuximab through EGFR-MET interaction and activation of MET signaling in colon cancer cells. Clin Cancer Res 2013;19:6751–65.
16. Ciardiello F, Bianco R, Caputo R, Damiano V, Troiani T, Melisi D, et al. Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. Clin Cancer Res 2004;10:784–93.
17. Choi Y, Syeda F, Walker JR, Finerty PJ, Cuerrier D, Wojciechowski A, et al. Discovery and structural analysis of Eph receptor tyrosine kinase inhibitors. Bioorg Med Chem Lett 2009;19:4467–70.
18. Ciardiello F, Normanno N, Martinelli E, Troiani T, Pisconti S, Cardone C, et al. Cetuximab continuation after first progression in metastatic colorectal cancer (CAPRI-GOIM): a randomized phase II trial of FOLFOX plus cetuximab versus FOLFOX. Ann Oncol 2016;27:1055–61.
19. Normanno N, Rachiglio AM, Lambiase M, Martinelli E, Fenizia F, Esposito C, et al. Heterogeneity of KRAS, NRAS, BRAF and PIK3CA mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial. Ann Oncol 2015;26:1710–4.
20. Zhuang G, Brantley-Sieders D, Vaught D, Yu J, Xie L, Wels S, et al. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Cancer Res 2010;70:299–308.
21. Macrae M, Neve RM, Rodriguez-Viciana P, Hagg C, Yeh J, Chen C, et al. A conditional feedback loop regulates Ras activity through EphA2. Cancer Cell 2005;8:111–8.
22. Brannan JM, Sen B, Saigal B, Prudkin L, Behrens C, Solis L, et al. EphA2 in the early pathogenesis and progression of non-small cell lung cancer. Cancer Prev Res 2009;12:1039–49.
23. De Robertis M, Loiacono L, Fusilli C, Poeta L, Mazza T, Sanchez M, et al. Dysregulation of EGFR pathway in EphA2 cell subpopulation significantly associates with poor prognosis in colorectal cancer. Clin Cancer Res 2017;23:159–70.
24. Karapetis CS, Khambata-Ford S, Jonker DJ, O' Callaghan CJ, Tebbutt NC, Simes RJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.