Circulating pEGFR Is a Candidate Response Biomarker of Cetuximab Therapy in Colorectal Cancer

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Abstract

Purpose: The lack of secreted biomarkers measurable by noninvasive tests hampers the development of effective targeted therapies against cancer. Our hypothesis is that cetuximab (an anti-EGFR mAb) induces a specific secretome in colorectal cancer cells that could be exploited for biomarker discovery.

Experimental Design: Considering the strong correlation between mutated KRAS and a lack of response to cetuximab therapy, we addressed whether performing secretome-based proteomics on isogenic colorectal cancer cells sharing the KRAS mutations found on patients would yield candidate-secreted biomarkers useful in the clinical setting. Because 2D culture did not optimally model the sensitivity/resistance to cetuximab observed in colorectal cancer patients, we moved to 3D spheroids, developing a methodology for both cell-based assays and quantitative proteomics.

Results: A large comparative quantitative proteomic analysis of the 3D secretomes of colorectal cancer isogenic cells treated with cetuximab uncovered an EGFR pathway-centric secretome found only when cells grow in 3D. The validation of the secretome findings in plasma of colorectal cancer patients, suggests that phosphorylated-EGFR (pEGFR) is a candidate-secreted biomarker of response to cetuximab.

Conclusions: We have proved that 3D spheroids from colorectal cancer cells generate secretomes with a drug-sensitivity profile that correlates well with patients with colorectal cancer, illustrating molecular connections between intracellular and extracellular signaling. Furthermore, we show how the secretion of pEGFR is associated with the sensitivity of colorectal cancer cells to cetuximab and the response of patients with colorectal cancer to the drug. Our work could allow the noninvasive monitoring of anti-EGFR treatment in patients with colorectal cancer. Clin Cancer Res; 20(24): 6346–56. ©2014 AACR.

Introduction

The role of EGFR in colorectal cancer initiation and progression is fundamental. EGFR-targeted therapies based on inhibiting the receptor tyrosine kinase activity and on blocking the ligand binding with mAbs are being used in the clinic. However, patients who initially benefit from EGFR-targeted therapies eventually develop resistance (1, 2). Intensive clinical trials have demonstrated a strong correlation between mutated KRAS and a lack of response to cetuximab therapy (3–11), though an interesting twist came recently when it was shown that not all the tumors carrying KRAS mutations were equally resistant to cetuximab action (12, 13).

In the clinic, the identification of specific and easily assayed secreted biomarkers for anti-EGFR drugs toward the prediction and monitoring of response and resistance to therapy is of paramount importance. The cancer secretome has emerged as a new approach for the proteomics-based investigation of tumorigenesis and responses to novel targeted therapies that avoid the limitations of blood-based profiling (14). Others and we have shown that cancer cell line secretomes consist of proteins that might help in monitoring critical aspects of cancer progression and therapeutics (15–17). Furthermore, secretome analysis has been used for comparative proteomics to study different aspects of cancer, particularly for tumor biomarker discovery (18, 19).

Preliminary research in our laboratory has shown that upon treatment, a cetuximab-specific secretome was induced in colorectal cancer cells during standard 2D cell culture conditions, obtaining statistically significant changes in the abundance of secreted proteins upon drug action. Therefore, we hypothesized that anti–EGFR-targeted drugs could induce protein secretion events, specific to drug response or resistance. Characterizing the cetuximab-induced protein secretion in colorectal cancer cells with the different mutational status for KRAS could further improve our understanding of the response and resistance
Translational Relevance

The role of EGFR in colorectal cancer initiation and progression is fundamental. EGFR-targeted therapies based on blocking the ligand binding with mAbs are being used in the clinic. The identification of specific and easily assayed secreted biomarkers for these anti-EGFR drugs toward the prediction and monitoring of response and resistance to therapy is of paramount importance. In this study, we describe an EGFR-centric secretome induced by cetuximab on 3D spheroids of colorectal cancer cells. Furthermore, we identify and preliminary validate in plasma of patients with colorectal cancer that phosphorylated-EGFR is a candidate-secreted biomarker of response to cetuximab. This work shows how intracellular and extracellular signaling are connected in tumor cells, and could allow the noninvasive monitoring of anti-EGFR treatment in patients with colorectal cancer.

mechanisms to the drug, and hint new strategies for biomarker discovery. We addressed our hypothesis by using isogenic colorectal cancer cell lines in which the KRAS status was precisely controlled. Parental [KRAS wild-type (WT)] SW48 cells were engineered using rAAV-mediated homologous recombination to create a panel of clones harboring different mutant KRAS variants at the endogenous locus, but otherwise shared the same genetic background. These endogenously engineered isogenic systems allow for unambiguous triangulation of specific genotype–phenotype interactions, while retaining the specific locus normal versus mutant context of target patients (20).

Considering the outcome of clinical data, we have focused on the KRAS-G12V and KRAS-G13D mutational statuses, because they are correlated with no response and limited response to cetuximab, respectively (12). 2D cell culture conditions proved not to be as robust as our 3D model in recapitulating the clinically observed sensitivity/resistance to cetuximab. Using 3D cell culture, we showed how the stimulation and blockage of the EGFR pathway in colorectal cancer cells are translated into an EGFR-centric protein secretion. Further validation of our secretome findings, in the plasma of patients undergoing cetuximab treatment, suggests that phosphorylated-EGFR (pEGFR) is a candidate-secreted biomarker of response to cetuximab.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Patient eligibility and study design

All patients (Supplementary Table S1) were diagnosed of metastatic colorectal cancer with the KRAS (exon 2) WT status and treated with the standard weekly schedule of cetuximab (400 mg/m² loading dose followed by 250 mg/m² weekly) in combination with the standard irinotecan-based cytotoxic regimen FOLFIRI (irinotecan 180 mg/m² day 1, leucovorin 400 mg/m² day 1, bolus 5-fluorouracil 400 mg/m² day 1, and infusional 5-fluorouracil 2,400 mg/m² in 48 hours starting day 1, repeated every 2 weeks), until progression of the disease or unacceptable toxicity. Response evaluation was planned every 8 weeks. All patients were treated in the first-line setting except for patient COLT016 that was treated in the second line after failing oxaliplatin-based chemotherapy (mFOLFOX6). Blood samples for the serum proteomic analysis were obtained at baseline (before the treatment was initiated), and thereafter every 8 weeks at the same time the response was evaluated.

Cell culture of differentiated cells and spheroids

The human colon adenocarcinoma cell line SW48 (KRAS WT) and the isogenic SW48 KI G12V and SW48 KI G13D (KRAS mutants) cells were obtained from Horizon Discovery Ltd. Horizon Discovery Ltd. authenticated the three cell lines used in this work by short tandem repeat profiling, and the cells were cultured in our laboratory for less than 6 months upon arrival. Cells have been tested for mycoplasma contamination by direct culture and Hoechst staining at Horizon Discovery Ltd., and by PCR using cell supernatants in our laboratory every 2 weeks during the experiments. Cells were cultured in 5% CO₂ and 95% humidified atmosphere at 37°C in DMEM: Nutrient Mixture F-12 (DMEM/F12; Invitrogen), supplemented with 10% FBS (Invitrogen), and 2 mmol/L L-glutamine (Invitrogen). For spheroids, SW48 cells meeting the viability limit of 90% were inoculated in T25 ultra-low attachment cell culture flasks (Corning Life Sciences) in a growth medium—colosphere medium—consisting of DMEM/F12, penicillin/streptomycin (100 U/100 µg/mL; Life Technologies), B27 (1:50; Gibco), heparin sodium salt (4 µg/mL), nonessential amino acids (1:100; Gibco), sodium pyruvate (1:100; Gibco), L-glutamine (2 mmol/L; Invitrogen), human recombinant FGF-2 and EGF (at 10 and 20 ng/mL, respectively; Peprotech), D-Glucose (60 mg/mL), apotransferrin (1 mg/mL), insulin (0.25 mg/mL), putrescin (96 µg/mL), sodium selenite (52 ng/mL), and progesterone (63 ng/mL).

On day 3, spheroids bloomed from clusters of cells with strong evidence of cell metabolism and two third of their culture medium was renewed, following low-speed centrifugation.

Secretome sample preparation in 2D and 3D

A detailed secretome preparation is provided in the Supplementary Section and illustrated in Supplementary Fig. S1. The downstream processing of the secretome samples and data for quantitative proteomic analysis is also outlined in Supplementary Fig. S1 and is described in great detail in the Supplementary Materials and Methods and in recent reports of our laboratory (16, 21).

Treatment regimen

Herein, both differentiated SW48 cells in 2D and 3D spheroids were treated with EGF (2 ng/mL), following an 18
hours serum starvation, at days 4 and 7, respectively. Their conditioned media were collected at 24 hours. For the cetuximab treatment condition, cells were treated with EGF (2 ng/mL; 10 min), followed by 0.5 mg/mL of cetuximab (days 4/7). After 24 hours, the cetuximab-treated cells were washed and their conditioned media were collected after 24 hours. Cell number and viability measurements were performed when the secretome was collected for each condition.

Western blotting

Differentiated SW48 cells and spheroids were seeded/ inoculated as described previously, and allowed to grow at the specified times and test conditions. Protein quantification and electrophoresis were performed as described elsewhere. Western blot analysis was performed using the rabbit polyclonal antibodies to pEGFR (Tyr1068; D7A5); p-p44/42 MAPK (T202/Y204; pErk1/2); and the p44/42 MAPK (ERK1/2)—all obtained from Cell Signaling Technology, Inc. The rabbit polyclonal antibodies for EGFR (1005 sc-03) and for phosphorilated AKT (Ser473; sc-7985-R) were obtained from Santa Cruz Biotechnology, Inc. The mouse anti-rabbit IgG horseradish peroxidase– conjugated secondary antibodies were purchased from GE Healthcare. Densitometry was carried out using the ImageJ software (NIH, Bethesda, MD).

pEGFR ELISA

The same amount of plasma samples from patients were used for pEGFR detection using the ELISA system (RayBio Phospho-EGFR (Tyr 1068) ELISA Kit) according to the manufacturer’s specifications.

Results

3D-spheroids of KRAS-mutated isogenic colorectal cancer cells model the sensitivity and resistance to cetuximab observed in patients with colorectal cancer

To establish a framework to characterize the cetuximab-induced protein secretion in colorectal cancer cells, we investigated the sensitivity or resistance of KRAS-WT versus KRAS-mutated isogenic colorectal cancer cell lines to cetuximab. We focused on isogenic cell lines in which the p.G12V and p.G13D alleles have been introduced in the genome of human colorectal SW48 cells by targeted homologous recombination (20). Both the EGF and cetuximab concentrations were optimized for our working conditions based on recently published studies (1, 12, 22); within a range of tested concentrations, we selected the lower ones that were still able to stimulate (EGF) and block (cetuximab) the proliferation of SW48 cells. To measure the stimulating and blocking effects on the EGFR pathway on SW48 cells, we calculated the percentage of cell proliferation in the EGF and the EGF/cetuximab conditions, normalized by the nontreated cells.

The two cell lines carrying the KRAS mutations showed a diminished response to EGF, perhaps because the EGFR pathway is constitutively active (Fig. 1A). The treatment with cetuximab resulted in a decrease of cell proliferation. Although the KRAS-G12V cells were less sensitive to the drug (Fig. 1A) than KRAS-G13D cells, they were still responsive; and because patients with colorectal cancer carrying the KRAS-G12V mutation do not respond to cetuximab treatment in the clinic, our results suggest that standard 2D cell culture conditions do not fully model well the correlation between the mutant KRAS and the resistance to the drug in vitro (12, 22). Hence, we applied the treatment regimens outlined above to all three isogenic cell lines, growing in 3D. In 3D, EGF similarly stimulates the proliferation of the three cell lines. However, only the proliferation of the KRAS-WT and KRAS-G13D cells is blocked by cetuximab. The proliferation of KRAS-G12V cells is not affected in the presence of cetuximab (Fig. 1B). The results obtained for the spheroids are in line with those obtained when the same cell lines were grown as xenografts in immunocompromised mice, and more importantly with the results obtained with patients with colorectal cancer (4, 10, 12, 13).

To evaluate whether the difference in the sensitivity to cetuximab of the KRAS-G12V cells could be due to the different media used in the 2D and 3D settings, instead of the adherent versus suspension culture conditions, we tested both SW48-WT and KRAS-G12V cells in DMEM/F12 supplemented with 10% FBS and 2 mmol/L L-glutamine versus the colonosphere medium in 2D upon treatment. The KRAS-G12V cells are resistant to cetuximab due to the cell culture format and not the media used for cell growth (Supplementary Fig. S2). To investigate further the sensitivity to cetuximab, both in 2D and 3D, we calculated the cell number upon treatment, normalized by the nontreated cells. Interestingly, the three cell lines are less sensitive to cetuximab in 3D than in 2D. KRAS-G12V cells become resistant to cetuximab most likely as a consequence of the global loss in sensitivity to the drug in 3D culture.

3D-secretomes of isogenic KRAS cells as a new platform to study the protein secretion induced by cancer drugs

Because our 3D spheroids model showed drug response profiles in 3D that better recapitulate patient data and differential secretome patterns compared with 2D conditions (Supplementary Fig. S3 and Supplementary Table S2), we next determined how drug treatment translates to a secreted molecular response. We profiled the secretomes of SW48 KRAS-WT, KRAS-G13D, and KRAS-G12V cells in response to EGF and EGF/cetuximab (in both 2D and 3D). Viability assays and the apoptotic analysis of cells show that SW48 cells maintain high cell viability and low apoptotic signal for 24 hours in serum-free media, which is not different than that obtained on the same cells growing with 10% serum.

The secretomes of the three cell lines (SW48 KRAS-WT, KRAS-G13D, and KRAS-G12V) upon treatment (EGF vs. cetuximab) are more dissimilar in 2D than in 3D (Fig. 2A and Supplementary Fig. S4). This observation is illustrated by the number of secreted proteins that are statistically significant.
significant when the two conditions (EGF and cetuximab) are compared (Fig. 2B and Supplementary Fig. S4). However, the 3D secretomes seem to be much more complex than those in 2D. In all three cell lines, the number of proteins identified in the 3D secretomes is consistently greater than in the corresponding 2D secretomes, for the same amount of secretome analyzed (Fig. 2A and Supplementary Fig. S4). Part of this additional complexity might come for an observed increase in exosomes present in the 3D secretome of the parental cell line, when compared with 2D (Fig. 2C; refs. 16, 23).

When we look at the unique and shared regulated proteins secreted in the three cell lines upon treatment, the isogenic cell line harboring the KRAS-G12V mutation has a large number of unique secreted proteins regulated in 3D spheroids as compared with the 2D cell culture. Supplementary Tables S3 and S4 refer to the gene ontology description of the encircled proteins shown in Fig. 2B. Because the KRAS-G12V mutation confers resistance to cetuximab in patients with colorectal cancer and we have observed a large decrease in sensitivity to cetuximab for this cell line growing in 3D, the large number of unique significant proteins secreted in the G12V mutant could be related to the change in drug sensitivity.

**Cetuximab action in SW48 cells differentially regulates the EGFR pathway–linked secreted response**

Because the sensitivity to cetuximab changes when the 2D setting is compared with the 3D spheroids, we reasoned that the EGFR pathway–related proteins could be differentially regulated in the secretomes of SW48 cells growing in the two culture formats. The analysis of the dataset related to the SW48 KRAS-WT cells upon treatment in 3D spheroids resulted in a list of 344 (Supplementary Table S5) differentially secreted proteins, when two biologic replicates are considered. This list was uploaded into the Ingenuity Pathway Analysis (IPA) software (see Supplementary Materials and Methods for details). The IPA core analysis showed that...
the top most significant network generated with our dataset contained two major hubs related to the EGFR pathway: EGFR and β-catenin (Fig. 3A). Because EGFR is the target of cetuximab and β-catenin is the main oncogenic driver in colorectal cancer initiation, the analysis shows the relevance of the secretome quantitative analysis toward the identification of response biomarkers to anti-EGFR therapy. Other proteins regulated in our model system and also previously
related to cetuximab treatment are c-Met, LIPAR, LAMC2, AREG, and TNC. Several of the proteins shown in the network have a direct implication to the EGFR pathway and might be candidate-secreted biomarkers of response to cetuximab therapy. To compare the molecular secreted response with cetuximab in 2D versus 3D, we checked whether the proteins regulated upon the action of cetuximab in spheroids were also regulated in 2D (Table 1, Supplementary Tables S3 and S6). Several proteins related to the EGFR pathway were only regulated in 3D spheroids or the regulation went to the opposite direction in 2D. The secretion of hundreds of proteins, among them growth factors (IAG1, HDGF, and MIF), proteases (pro-cathepsin H), and extracellular matrix proteins (laminins A5, B2, B3 and C2), was regulated, when the KRAS-WT cells were treated with cetuximab in 2D, but no clear canonical pathways were enriched, and no direct relationship to the EGFR pathways was established.

Next, we compared the levels of EGFR by Western blot analysis in the secretomes of SW48 cells cultured in 2D or in 3D, confirming the oversecretion of EGFR in the secretomes of 3D spheroids SW48-WT cells upon the treatment with cetuximab (Fig. 3B). Interestingly, we also found that a fraction of the EGFR oversecreted is the Tyr1068-phosphorylated form of EGFR (pEGFR). Because we are able to detect peptides from the intracellular domain of the receptor, we suggest that the full receptor is secreted through extracellular vesicles. This would be in agreement with the fact that we detect a stronger signal for exosomes in 3D spheroids (Fig. 3C). To evaluate the EGFR intracellular activation status in the two cell culture formats, we profiled the activated form of three major nodes in the EGFR pathway (pEGFR, pERK, and pAKT). In 3D, the modulation of the pEGFR when it is stimulated with EGF and blocked by cetuximab is more finely regulated than in 2D. Furthermore, although pAKT is almost constitutively active and not regulated in both 2D and 3D, the basal level of pERK is higher in 3D than in 2D, and gets completely blocked upon cetuximab treatment (Fig. 3C). Finally, to compare the pEGFR secretion between sensitive and resistant cells, we graphed the mass spec measurements of SW48 parental and KRAS-G12V cells grown in 3D, and treated either with EGF or with EGF and cetuximab (Fig. 3D). The results clearly show that the receptor is being secreted upon cetuximab treatment in the sensitive cells. Therefore, the secretome and pathway activation molecular findings confirm that there is an EGFR-centric differential secretion in line with sensitivity to cetuximab, depending on the cell culture format. This observation has potential implications for biomarker discovery in the context of anti–EGFR-targeted drugs.

Secreted pEGFR is a candidate biomarker of response to cetuximab

Next, we performed a clinical validation measuring pEGFR in plasma by ELISA of patients with colorectal cancer undergoing treatment with cetuximab plus FOLFIRI (Supplementary Table S1). The only requirement to receive the treatment for these patients is being KRAS wt. We took plasma samples from patients before treatment and every 8 weeks during the treatment. Here, we present data for 18 patients with a baseline sample and at least two samples collected during the treatment. All the treatment samples have been categorized as partial response (PR), stable disease (SD), or progressive disease (PD) by radiologic assessment following the RECIST criteria. The results show that the samples obtained during the anti-EGFR treatment, and classified as PR present, increased pEGFR levels compared with baseline samples. SD samples show no changes in pEGFR, whereas PD samples show decreasing pEGFR levels than obtained for baseline samples (Fig. 4A). In 13 patients of the 18 patients (patients with green labels in Fig. 4A), the circulating levels of pEGFR correlate with the treatment outcome and suggest that pEGFR is a candidate biomarker of drug response (Fig. 4A). Noteworthy, 2 of the patients (COLT019 and COLT030) had a BRAF-activating mutation. Patient COLT019 did not respond to the treatment (and showed no changes in pEGFR levels) as expected. However, patient COLT030 had some variation of pEGFR levels but based on the BRAF mutation we cannot make any judgment on this behavior. For 5 patients (patients with red labels in Fig. 4A), the pEGFR levels during treatment do not seem to be associated with the treatment outcome and currently we do not have an explanation for these experimental observations. However, on the basis of the results obtained with the patients with BRAF mutations, we expect that these patients have hyperactivation of the EGFR pathway downstream of the receptor or in other related pathways, which would uncouple the secretion of pEGFR from the response to the cetuximab treatment.

Finally, we show the CT scan images performed on 2 of the patients shown in Fig. 4A and B. The patient COLT014, who responds to the treatment, shows a clear reduction of the neoplastic lesions, whereas in the patient with a BRAF mutation (COLT019) cetuximab treatment has no effect on the tumor lesions. The clinical response to cetuximab assessed by CT scans is mirrored by the trend in plasma levels of pEGFR. Therefore, our results suggest that circulating pEGFR is a candidate biomarker of drug response, although more work is needed to assess the full potential of this biomarker.

Discussion

A major obstacle in the treatment of patients with cancer is the lack of secreted biomarkers of response and primary/secondary resistance to therapy that could be monitored by noninvasive tests. In the particular case of colorectal cancer and anti-EGFR mAb therapy, there is a strong correlation between KRAS and BRAF mutations (found to be mutually exclusive) and a lack of response to cetuximab therapy. Given that KRAS and BRAF mutations are present in approximately 50% of colorectal cancer advanced tumors, and that these mutations can also be acquired by tumors because of therapeutic treatment, the identification of useful biomarkers for anti-EGFR treatment could have a significant clinical impact. The search for secreted biomarkers that can guide the therapeutic treatment of choice is typically
Figure 3. The cetuximab action in SW48 cells differentially regulates the EGFR pathway–linked secreted response in 3D spheroids as compared with the 2D cell culture. A, IPA of the proteins that are differentially secreted upon EGF and EGF/cetuximab treatment of SW48 parental cells in 3D (two biologic replicates and three technical replicates). (Continued on the following page.)
Table 1. Selected proteins differentially secreted upon Cetuximab treatment in 2D and 3D cell culture

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Name</th>
<th>3D</th>
<th>Adj-LogFC</th>
<th>Adj-P value</th>
<th>2D</th>
<th>Adj-LogFC</th>
<th>Adj-P value</th>
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<td>Epidermal growth factor receptor</td>
<td>EGFR</td>
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<td>1.7</td>
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<td>9.3</td>
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<td>Frataxin, mitochondrial</td>
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<td>11.3</td>
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<td>9.3</td>
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<td>Chromodomain-helicase-DNA-binding protein 4</td>
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<td>CTNNB1</td>
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<td>2.84</td>
<td>3.86E-03</td>
<td>0.7</td>
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a Numeric values correspond to normalized spectral counts (ref. 16,21)
b Fold changes and adjusted p-values were calculated from the GLM Poisson modelling of the data (see Suppl. Material and Methods)

(Continued.) The network contains secretome proteins (in red and green) and gene objects (in white) connected to them by IPA. Green, secretome proteins whose levels increase upon EGF treatment, whereas red corresponds to secretome proteins whose levels increase upon cetuximab treatment in 3D. B, Western blot analysis of secreted pEGFR in 2D and 3D cultured SW48-WT cells. Equal concentrations (15 μg) of secretomes were resolved by SDS-PAGE and Western blotted against pEGFR(Y1068) and total EGFR. C, Western blot analysis of cell lysates showing the activation of the EGFR pathway. Cells grown in 2D or 3D were starved for 18 hours and stimulated with EGF (2 ng/mL; 10 minutes), or equally stimulated with EGFR followed with 3 hours of cetuximab treatment (0.5 mg/mL). Equal concentrations (20 μg) of cell lysates were resolved by SDS-PAGE and Western blotted against pEGFR(Y1068), total EGFR, pAKT(S473), pERK1/2, total ERK1/2, and α-Tubulin, as a loading control. D, mass spectrometric signal (measured by spectral counts) of EGFR in SW48 cells. Graph, data from two biologic replicates (each having three technical replicates) of both SW48 parental cells and SW48 cells containing a KRAS-G12V allele. The two cell lines were treated with either EGF or with EGF and cetuximab as described in the experimental procedures.
cetuximab with respect to their *KRAS* mutational status. Then, we showed how the stimulation and blockage of the EGFR pathway in colorectal cancer cells is translated into an EGFR-centric protein secretion. Finally, the preliminary validation of our findings in the plasma of patients with colorectal cancer suggests that the level of secreted pEGFR correlates with the response to cetuximab treatment.

Our studies prove that 3D spheroids are better able to model the sensitivity/resistance to cetuximab according to the *KRAS* mutational status, as previously observed in xenografts and patients with colorectal cancer (12,22). We were able to more robustly model the partial sensitivity of the *KRAS*-G13D mutation in 3D, which opened up a new therapeutic avenue previously closed for these patients with colorectal cancer. The 3D spheroids, but not the 2D cell culture format, set apart the two *KRAS* mutations studied herein under the drug exposure. The relevance of the 3D cultures for cancer research has been previously underscored, as it represents a more physiologic approach allowing a better modeling of the therapeutic treatment investigated than in 2D (24–26). Herein, the SW48 isogenic cell lines are overall less sensitive to cetuximab in 3D than in 2D, and clinically important differences based on the *KRAS* mutational variant status were now revealed. This is even reflected at the secretome level, in which the number of the significant differences arising from the secretome comparison upon treatment with cetuximab in the three cell lines is much smaller in spheroids than in 2D. More important, the treatment of colorectal cancer cells with cetuximab in 2D showed a broad effect on different signaling pathways, but most of them not directly related to the EGFR pathway. On the contrary, the cetuximab-treated *KRAS-WT* cells growing as 3D spheroids showed a clear regulation in protein secretion related to the EGFR pathway. Several of the molecules...
regulated in the 3D spheroids in the presence of cetuximab are regulated in patients treated with anti–EGFR-targeted therapy either in colorectal cancer or lung cancer (27–28). Although it is difficult to explain why cells would respond differently to drugs in 3D, it has been established that changes in cell geometry and organization can directly affect cell function (29) and noteworthy, the penetration, binding, and bioactivity of therapeutic drugs (30).

A remarkable finding in this work is that pEGFR is secreted from colorectal cancer cells treated with cetuximab, particularly in the 3D spheroid setting. This finding coincides with an increased amount of exosomes in the colorectal cancer secretome. We hypothesize that shedded vesicles containing pEGFR could be used by colorectal cancer cells as an escape strategy for the negative effects of cetuximab on cell viability, as it has also been proposed for ERBB2 and trastuzumab therapy (31). However, in glioblastoma cells, although the shedding of extracellular vesicles carrying a mutant form of the EGFR (EGFRvIII) seems to stimulate angiogenesis, these vesicles have not been linked to cancer therapy (32–33). The fact that the secretion of pEGFR upon cetuximab treatment is more stimulated in KRAS-WT cells than KRAS-mutant cells could be related to the diminished importance of EGFR once the pathway is constitutively activated via KRAS mutations. This observation could explain why in some patients we see a decrease of pEGFR secretion once they progress, most likely because they acquire hyperactivating mutations of the pathway.

Previously, the tissue-activated form of EGFR has been reported to be a candidate response biomarker of cetuximab in metastatic colorectal cancer. Personeni and colleagues (34) found in a small-scale study that the level of tissue EGFR activation measured by immunohistochemistry was associated with the sensitivity to cetuximab in mCRC. However, in that study the mutational status of downstream effectors such as KRAS and BRAF of EGFR was not taken into account, and no follow-up of the initial results has been done. Our work instead focuses on how the blockage of the EGFR pathway in sensitive cell to cetuximab is translated into the oversecretion of pEGFR. Overall, the preliminary clinical validation of our secretome findings suggests that circulating pEGFR is a candidate biomarker of cetuximab response. Most of the 18 patients show pEGFR plasma levels that correlate with the treatment outcome followed by standard radiologic assessment. However, more patients have to be added to our pilot study, and independent studies have to confirm our results. Furthermore, we need a mechanistic insight that helps to understand why in some cases pEGFR plasma levels would not correlate with therapeutic treatment. Probably, the hyperactivation of the EGFR pathway due to downstream activating mutations or mutations in other pathways such as PI3K pathway is likely to influence in the secretion levels of pEGFR.

Blood-based tumor biomarker discovery has proven to be extremely difficult due to the complexity and large dynamic range of protein concentrations in plasma, which is further exacerbated by the relatively low abundance of tumor-specific biomarkers. The secretome approach seems a convincing alternative for biomarker discovery once a clinically relevant model system is established. Herein, we have proved that the spheroids generating the 3D secretomes have a drug-sensitivity profile that correlates well with xenografts and patients with colorectal cancer, illustrating molecular connections between intracellular and extracellular signaling that are potentially relevant for cancer diagnostics and therapeutics. Furthermore, we show that the secretion of pEGFR is associated with the sensitivity of colorectal cancer cells to cetuximab and with the response of patients with colorectal cancer to the drug. Our work could allow the noninvasive monitoring of cetuximab treatment in patients with colorectal cancer, although to further determine its true clinical potential, a large blinded independent study will be needed.

Disclosure of Potential Conflicts of Interest
J. Villanueva, T. Katsila, and J. Tabernero are coinventors of a pending patent on the use of circulating pEGFR as a biomarker of response to cetuximab therapy, which was filed with the European patent office by Vall d’Hebron Institute of Oncology, the authors’ institution. A. Bardelli has ownership interest (including patents) in and is an uncompensated consultant/advisory board member for Horizon Discovery. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: T. Katsila, M. Juliachs, J. Gregori, J. Villanueva
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Katsila, M. Juliachs, T. Macarulla, E. Elez, J. Tabernero
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): T. Katsila, M. Juliachs, J. Gregori, T. Macarulla, J. Tabernero, J. Villanueva
Writing, review, and/or revision of the manuscript: T. Katsila, M. Juliachs, J. Gregori, T. Macarulla, J. Tabernero, J. Villanueva
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Juliachs, T. Macarulla, J. Villanueva
Study supervision: T. Macarulla, J. Villanueva

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