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Running title: *pEGFR is a response biomarker of cetuximab therapy in CRC*

Abstract

Purpose: The lack of secreted biomarkers measurable by non-invasive 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 (CRC) 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 CRC cells sharing the KRAS-mutations found on patients would yield candidate secreted biomarkers useful in the clinical setting. Since 2D-culture did not optimally model the sensitivity/resistance to cetuximab observed in CRC 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 CRC 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 CRC patients, suggests that phosphorylated-EGFR (pEGFR) is a candidate secreted biomarker of response to cetuximab.

Conclusions: We have proved that 3D-spheroids from CRC cells generate secretomes with a drug sensitivity profile that correlates well with CRC patients, illustrating molecular connections between intracellular and extracellular signaling. Furthermore, we show how the secretion of pEGFR is associated with

the sensitivity of CRC cells to cetuximab and the response of CRC patients to the drug. Our work could allow the non-invasive monitoring of anti-EGFR treatment in CRC patients.

Translational Relevance

The role of Epidermal Growth Factor Receptor (EGFR) in colorectal cancer (CRC) initiation and progression is fundamental. EGFR-targeted therapies based on blocking the ligand binding with monoclonal antibodies are being used in the clinic. The identification of specific and easily assayed secreted biomarkers for these anti-EGFR drugs towards 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 CRC cells. Furthermore, we identify and preliminary validate in plasma of CRC patients that phosphorylated-EGFR (pEGFR) 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 non-invasive monitoring of anti-EGFR treatment in CRC patients.

Key words: Secretome, anti-EGFR drugs, 3D-culture, cetuximab, pEGFR

Introduction

The role of Epidermal Growth Factor Receptor (EGFR) in colorectal cancer (CRC) initiation and progression is fundamental. EGFR-targeted therapies based on inhibiting the receptor tyrosine kinase activity and on blocking the ligand binding with monoclonal antibodies 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 towards 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 lab has shown that upon treatment, a cetuximab-specific secretome was induced in CRC 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 CRC cells with different mutational status for KRAS could further improve our understanding of the response and resistance mechanisms to the drug, and hint new strategies for biomarker discovery. We addressed our hypothesis by using isogenic CRC cell lines in which the KRAS status was precisely controlled. Parental (KRAS wild-type) SW48 cells were engineered using rAAV-mediated homologous recombination to create a panel of clones harbouring 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, since they are correlated to 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 CRC cells is 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 (Madrid, Spain) unless stated otherwise.

Patient eligibility and study design

All patients (Supplemental Table S1) were diagnosed of metastatic colorectal cancer with K-RAS (exon 2) wild type status and treated with the standard weekly schedule of cetuximab (400 mg/m^2 loading dose followed by 250 mg/m^2 weekly) in combination with the standard irinotecan-based cytotoxic regimen FOLFIRI (irinotecan 180 mg/m^2 day 1, leucovorin 400 mg/m^2 day 1, bolus 5-fluoruracil 400 mg/m^2 day 1 and infusional 5-fluorouracil 2400 mg/m^2 in 48 hours starting day 1, repeated every 2 weeks), until progression of the disease or unacceptable toxicity. Response evaluation was planned every eight 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 eight weeks at the same time the response was evaluated.

Cell culture of differentiated cells and spheroids. The human colon adenocarcinoma cell line SW48 (*KRAS*-wild type) 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 STR profiling, and the cells were cultured in our laboratory for less than six 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 two weeks during the experiments. Cells were cultured in 5% CO_2 and 95% humidified atmosphere air at 37°C in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), and 2 mM L-Glutamine (Invitrogen, Carlsbad, CA). For spheroids, SW48 cells meeting the viability limit of 90% were inoculated in T25 ultra-low attachment cell culture flasks (Corning Life Sciences, Aston, MA) in a growth medium – colonosphere medium – consisting of DMEM/F12, penicillin/streptomycin (100U/100 µg/mL; Life Technologies, Carlsbad, CA), B27 (1:50; Gibco), heparin sodium salt (4 µg/mL), non-essential amino acids (1:100; Gibco), sodium pyruvate (1:100; Gibco), L-Glutamine (2mM; Invitrogen, Carlsbad, CA), human recombinant FGF-2 and EGF (at 10 and 20 ng/mL, respectively; Peprotech, Rocky Hill, NJ), 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-thirds 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 Figure S1.

The downstream processing of the secretome samples and data for quantitative proteomic analysis is also outlined in Figure 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 18h serum starvation, at day 4 and day 7 respectively. Their conditioned media were collected at 24 h. For the cetuximab treatment condition, cells were treated with EGF (2 ng/mL, 10 min), followed by 0.5 mg/mL of cetuximab (day 4/ day 7). After 24h, the cetuximab-treated cells were washed and their conditioned media were collected after 24h. Cell number and viability measurements were performed when the secretome was recollected for each condition.

Western Blotting. Differentiated SW48 cells and spheroids were seeded/ inoculated as described, and allowed to grow at the specified times and test-conditions. Protein quantification and electrophoresis was performed as described elsewhere. Western blot analysis was performed using the rabbit polyclonal antibodies to phosphorylated -EGFR (Tyr1068) (D7A5); p–p44/42 MAPK (T202/Y204) (pErk1/2) and the p44/42 MAPK (ERK1/2)- all obtained from Cell Signaling Technology, Inc. (Danvers, MA). The rabbit polyclonal antibodies for EGFR (1005 sc-03), and for phosphorilated AKT (Ser473) (sc-7985-R) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz-CA). The mouse

monoclonal antibody to TSG101 was purchased from Abcam (Cambridge, UK). Sheep anti-mouse and donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (Amersham, UK). Densitometry was carried out using the Image J software (National Institutes of Health, Bethesda, MD).

Phosphorylated EGFR ELISA.

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

RESULTS

3D-spheroids of KRAS-mutated isogenic CRC cells model the sensitivity and resistance to cetuximab observed in CRC patients. To establish a framework to characterize the cetuximab-induced protein secretion in CRC cells we investigated the sensitivity or resistance of KRAS-wild type versus KRAS-mutated isogenic CRC cell lines to cetuximab. We focused on isogenic cell lines where 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 non-treated cells.

The two cell lines carrying the KRAS mutations showed a diminished response to EGF, perhaps because the EGFR pathway is constitutively active (Figure 1A). The treatment with cetuximab resulted in a decrease of cell proliferation. Although the KRAS-G12V cells were less sensitive to the drug (Figure 1A) than KRAS-G13D cells, they were still responsive; and since CRC patients 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-wild type and KRAS-G13D cells is blocked by cetuximab. The proliferation of KRAS-G12V cells is not affected in the presence of cetuximab (Figure 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 CRC patients (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 wild-type and KRAS-G12V cells in DMEM/F12 supplemented with 10% FBS and 2 mM 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 (Figure S2). To investigate further the sensitivity to cetuximab, both in 2D and 3D, we calculated the cell number upon treatment, normalized by the non-treated 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. Since our 3D-spheroids model

showed drug response profiles in 3D that better recapitulate patient data and differential secretome patterns compared to 2D-conditions (Figure S3 and Supplemental Table S2), we next determined how drug treatment translates to a secreted molecular response. We profiled the secretomes of SW48 KRAS-wild type, 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 24h 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-wild type, KRAS-G13D and KRAS-G12V) upon treatment (EGF vs. Cetuximab) are more dissimilar in 2D than in 3D (Figures 2A, S4). This observation is illustrated by the number of secreted proteins that are statistically significant when the two conditions (EGF and cetuximab) are compared (Figures 2B, 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 (Figures 2A, 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 to 2D (Figure 2C) (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 to the 2D-cell culture. The Supplemental table S3 and S4 refer to the gene ontology description of the encircled proteins shown in Figure 2B. Since, the KRAS-G12V mutation confers resistance to cetuximab in CRC patients 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. Since the sensitivity to cetuximab changes when the 2D setting is compared to the 3D-spheroids, we reasoned that 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-wild type cells upon treatment in 3D-spheroids resulted in a list of 344 (Supplemental Table S5) differentially secreted proteins, when two biological 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 beta-catenin (Figure 3A). Since EGFR is the target of cetuximab and beta-catenin is the main oncogenic driver in CRC initiation, the analysis shows the relevance of the secretome quantitative analysis towards 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,

UPAR, 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 to cetuximab in 2D versus 3D, we checked whether the proteins regulated upon the action of cetuximab in spheroids were also regulated in 2D (Tables 1, Supplemental Table S5 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 (JAG1, HDGF, MIF), proteases (pro-cathepsin H) and extracellular matrix proteins (laminins A5, B2, B3 and C2) was regulated, when the KRAS-wild type 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 in the secretomes of SW48 cells cultured in 2D or in 3D, confirming the oversecretion of EGFR in the secretomes of 3D-spheroids SW48 wild-type (WT) cells upon the treatment with cetuximab (Figure 3B). Interestingly, we also found that a fraction of the EGFR oversecreted is the Tyr1068-phosphorylated form of EGFR (pEGFR). Since 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 (Figure 2C). 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, 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 while 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 (Figure 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 (Figure 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 CRC patients undergoing treatment with cetuximab plus FOLFIRI (Supplemental 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 (BL) 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 to BL samples. SD samples show no changes in pEGFR, whereas PD samples show decreasing pEGFR levels than obtained for BL samples (Figure 4A). In thirteen patients out of the eighteen patients (patients with green labels in figure 4A), the circulating levels of pEGFR correlate with the treatment outcome and suggest that pEGFR is a candidate biomarker of drug response (Figure 4A). Noteworthy, two 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 five patients (patients with red labels in figure 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, based on 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 two of the patients shown in figure 4A (Figure 4B). The patient COLT014, who responds to the treatment, shows a clear reduction of the neoplastic lesions, while 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 cancer patients is the lack of secreted biomarkers of response and primary/secondary resistance to therapy that could be monitored by non-invasive tests. In the particular case of CRC 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 CRC 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 performed using *in vitro* experimental model systems, due to the plasma complexity. However, it is difficult to establish how relevant for the disease is the experimental model system used *in vitro*. The work reported here was aimed at the characterization of the protein secretion involved in the response and resistance to cetuximab therapy, and its exploitation for biomarker discovery. A

3D-spheroid methodology was developed and made compatible with secretome analysis, showing that protein secretion in 3D differs to that in 2D, modeling best the sensitivity of patients to cetuximab with respect to their KRAS mutational status. Then, we showed how the stimulation and blockage of the EGFR pathway in CRC cells is translated into an EGFR-centric protein secretion. Finally, the preliminary validation of our findings in the plasma of CRC patients 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 CRC patients (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 CRC patients. 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, where 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 CRC 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-wild type 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 CRC 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 impact 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 CRC cells treated with cetuximab, particularly in the 3D-spheroid setting. This finding coincides with an increased amount of exosomes in the CRC secretome. We hypothesize that shedded vesicles containing pEGFR could be used by CRC 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 CRC (34). Personeni et al. 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 over-secretion 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 eighteen 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 are 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 CRC patients, 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 CRC cells to cetuximab and with the response of CRC patients to the drug. Our work could allow the non-invasive monitoring of cetuximab treatment in CRC patients, although to further determine its true clinical potential, a large blinded independent study will be needed.

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TABLES

Table 1. Selected proteins differentially secreted upon Cetuximab treatment in 2D and 3D cell culture

		3D				2D			
Protein	**Gene Name**	**Cetuximab**	**EGF**	**LogFC**	**Adj-pvalue**	**Cetuximab**	**EGF**	**LogFC**	**Adj-pvalue**
Epidermal growth factor receptor	EGFR	5	0	31.86	1.16E-05				
Calnexin	CANX	10.7	1.7	2.93	2.36E-06				
Solute carrier family 12 member 2	SLC12A2	11.7	2.7	2.38	1.28E-05	14.3	9.3	2.40	1.75E-11
Dipeptidyl peptidase 2	DPP7	18.7	5	2.15	1.58E-07	6.7	13	0.86	0.04426
Frataxin, mitochondrial	FXN	17.7	5	2.07	6.81E-07				
Superoxide dismutase [Mn], mitochondrial	SOD2	81.3	27.7	1.81	1.68E-24	11	11	1.74	3.00E-06
Four and a half LIM domains protein 2	FHL2	29.3	11	1.67	3.01E-08				
60 kDa heat shock protein, mitochondrial	HSPD1	367.7	155	1.50	3.98E-84	104.3	108	1.73	1.75E-47
Apoptosis regulator BAX	BAX	12.7	5.7	1.41	0.002215				
Cathepsin L2	CTSL2	89.3	47.3	1.17	4.01E-14				
Yorkie homolog	YAP1	56.7	34	0.99	3.71E-07	8.7	9	1.73	3.76E-05
Amyloid beta A4 protein	APP	36	75.3	-0.82	6.77E-06				
Laminin subunit gamma-2	LAMC2	36.3	90.3	-1.06	2.72E-10	7.7	94	-1.84	4.91E-12
FACT complex subunit SPT16	SUPT16H	13.7	36.3	-1.16	3.23E-05	28	7	3.78	1.01E-34
Bone morphogenetic protein 4	BMP4	6.3	18	-1.26	0.002554				
Urokinase plasminogen activator surface receptor	PLAUR	12	34.7	-1.28	1.06E-05	0	11	-32.41	6.07E-05
Importin subunit alpha-2	KPNA2	9.3	27	-1.28	0.0001235				
Hepatocyte growth factor receptor	MET	4	11.7	-1.29	0.01514	1	28	-3.04	5.63E-07
Tenascin	TNC	9	26.7	-1.32	9.72E-05	0	28	-32.32	1.45E-10
FACT complex subunit SSRP1	SSRP1	13	42.7	-1.46	3.93E-08	29.7	12	3.13	2.37E-30
Amphiregulin	AREG	10.7	35.7	-1.49	4.12E-07	3.7	35	-1.47	0.0003067
Nucleolin	NCL	121.7	409	-1.50	1.01E-76	137.7	98	2.27	8.45E-92
General vesicular transport factor p115	USO1	11	38.3	-1.55	5.76E-08				
Nucleophosmin	NPM1	69	247	-1.59	1.92E-50	70	69	1.80	5.44E-34
Insulin-like growth factor-binding protein 4	IGFBP4	3	11.3	-1.67	0.003235				
Extracellular matrix protein FRAS1	FRAS1	5	21	-1.82	9.15E-06				
Alpha-1-antitrypsin	SERPINA1	5.7	26.7	-1.98	8.32E-08	1	30	-3.14	1.51E-07
E3 SUMO-protein ligase RanBP2	RANBP2	3	19.3	-2.44	2.35E-07	9.7	2.7	3.64	1.77E-12
Chromodomain-helicase-DNA-binding protein 4	CHD4	2	13	-2.45	2.73E-05				
Catenin beta-1	CTNNB1	0.7	5.7	-2.84	0.003857				

FIGURE LEGENDS

Figure 1. Cetuximab effect on cellular proliferation of SW48 KRAS-wild type and KRAS-mutant isogenic cell lines in 2D and 3D cell culture. **A**. Cetuximab inhibits the proliferation of both KRAS-wild type and KRAS-mutant cell lines in 2D. KRAS-wild type, KRAS-G12V and KRAS-G13D cells were serum starved for 18h and treated with 2 ng/mL of EGF or 2 ng/mL of EGF (10 min) and 0.5 mg/mL of cetuximab for 24h. Non-treated cells served as negative controls for each cell line. Cell viability and % proliferation were determined using Countess (Invitrogen, Carlsbad, CA). Results were normalized to growth of non-treated cells. Experiments were performed, at least, in triplicate. Results are shown as mean ± std. **B.** Cetuximab inhibits the proliferation of KRAS-wild type and KRAS-G13D cell lines, but does not affect the KRAS-G12V cell line in 3D. As described for the 2D setting, KRAS-wild type, KRAS-G12V and KRAS-G13D cells were treated with EGF or EGF/cetuximab, following serum starvation. Non-treated cells also served as negative controls for each cell line. Cell viability and % proliferation were determined and results were normalized to growth of non-treated cells. Experiments were performed, at least, in triplicate. Results are shown as mean ± std. EGF = epidermal growth factor; wt = KRAS-wild type cells; G12V = KRAS-G12V cells; G13D = KRAS-G13D cells

Figure 2. Secretome Proteomics of Sensitivity to Cetuximab in 2D and 3D. **A.** Heat maps representing the proteins that were significantly over- and/or down secreted, when SW48 parental, KRAS-G13D and KRAS-G12V cells were grown

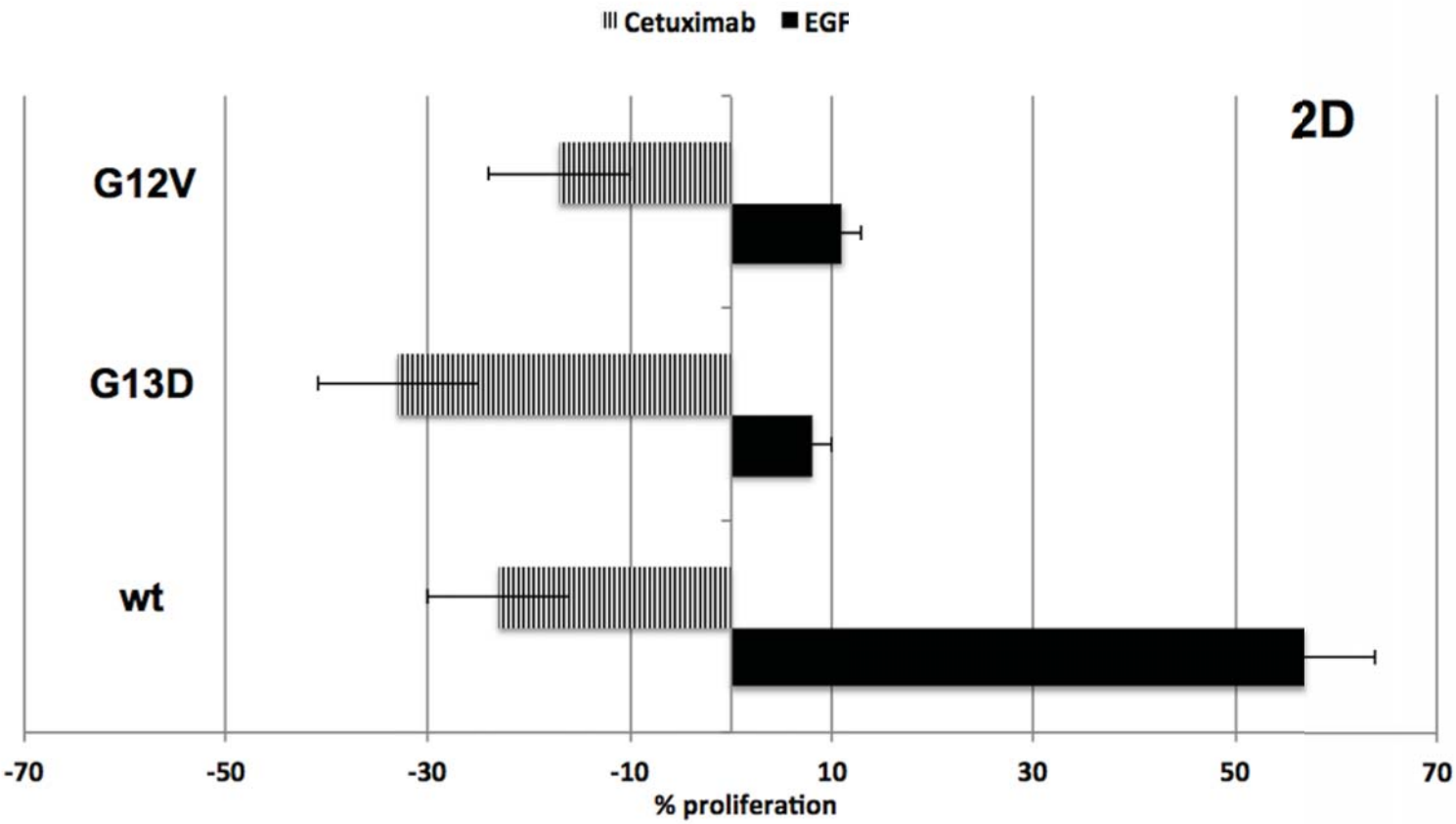
in the 2D or 3D format and treated with EGF or EGF/cetuximab (first biological replicate). Data analysis was based on spectral count data after exporting it from Scaffold software into R. The GLM model based on the Poisson distribution was used to test significance. The Benjamini & Hochberg multitest correction was employed to adjust the p-values with control on the FDR. The data were normalized on the basis of secreted protein per cell, taking also into account an adjusted significance value of 0.05 and the post-test filters (SpC of 4, LogFC of 0.8), as described in the Experimental procedures section. A high abundance filter of 11 was used on the entire protein list exported from Scaffold software. Columns represent samples; rows are proteins. Red represents proteins that were over-secreted and green represents proteins that were down-secreted in the presence of EGF or EGF/cetuximab. The data rows are centered and scaled to 1 sd prior to produce the heatmap. **B.** Venn diagrams showing the significant proteins that differ between the two cell culture formats (2D and 3D) upon cetuximab treatment, when comparing the dataset of KRAS-wild type status to the dataset of KRAS-G13D and that of KRAS-G12V status. The GLM Poisson test was used for the generation of the Venn diagrams. **C.** Equal concentrations of secretomes (15 μg) were resolved by SDS-PAGE and western blotted against TSG101, an established exosome marker. TSG101 levels are obtained in the 3D format, but not in 2D, showing differences among treatments and when compared to the non-treated cells.

Figure 3. The cetuximab action in SW48 cells differentially regulates the EGFR pathway-linked secreted response in 3D-spheroids as compared to the 2D-cell culture. A. Ingenuity Pathway Analysis of the proteins that are differentially secreted upon EGF and EGF/ cetuximab treatment of SW48 parental cells in 3D (two biological replicates, three technical replicates). The network contains secretome proteins (in red and green) and gene objects (in white) connected to them by IPA. Green represents 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 18h and stimulated with EGF (2ng/ml; 10 min), or equally stimulated with EGF followed with 3h 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),pERK 1/2, total ERK1/2 and α-tubulin, as a loading control. **D.** Mass spectrometric signal (measured by spectral counts) of EGFR in SW48 cells. The graph contains data from two biological 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.

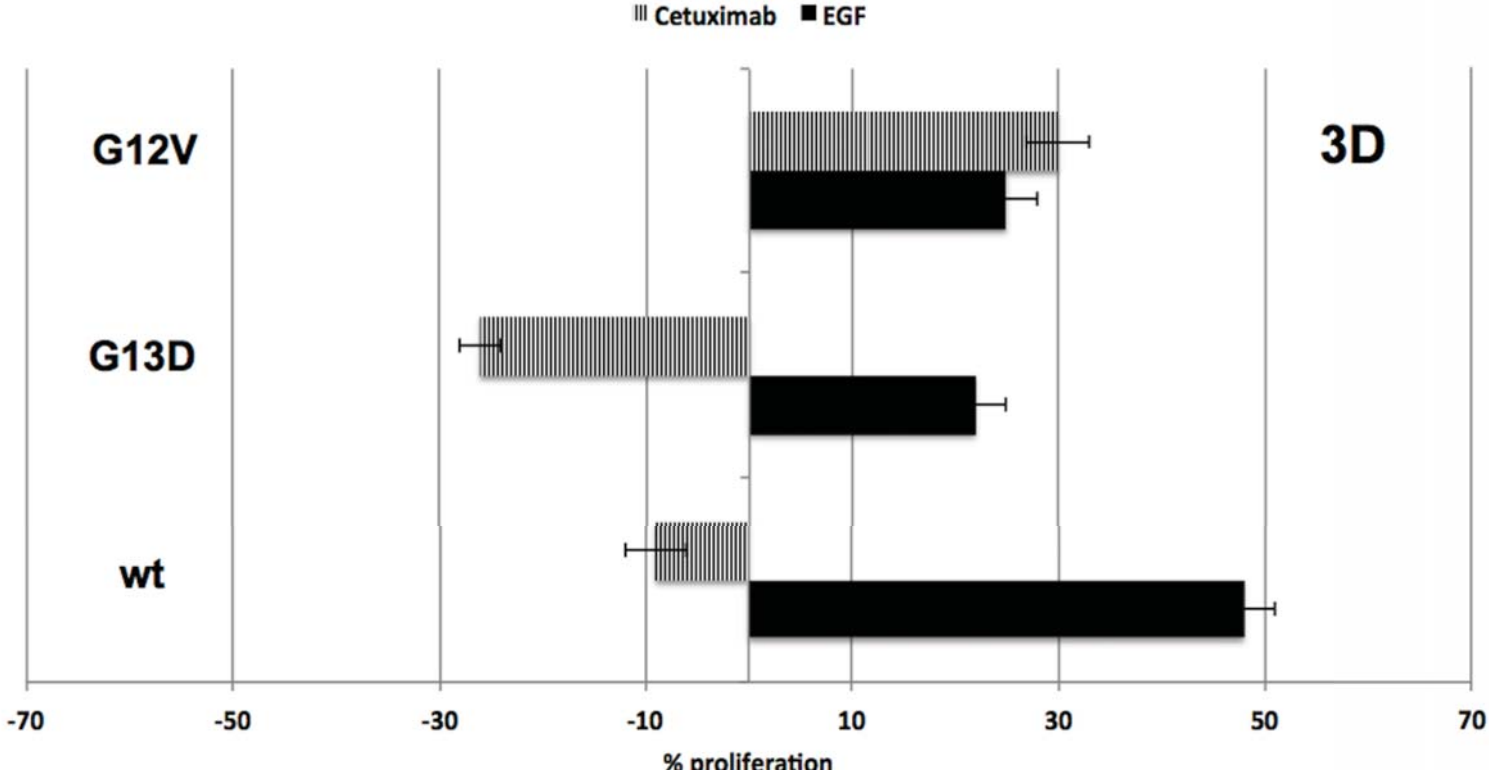
Figure 4. pEGFR secretion is validated in secretomes and in the plasma of patients undergoing cetuximab treatment. A. pEGFR levels were detected by ELISA in the plasma of patients undergoing cetuximab treatment. The same amount of plasma (100µl) was analyzed for each patient sample. Results are shown as mean ± std. Experiments were performed, at least, in triplicate. RP = partial response to cetuximab; SD = stable disease; PD = progressive disease. The labeled-disease status during treatment (RP, SD, PD) was assessed by standard CT scan-based imaging. Note that the PD sample for patient COLT009 has higher pEGFR levels than at baseline but lower levels than at maximum response. **B** CT scans of patients COLT014 and COLT019. The imaging of the two patients is shown before (top panels) and after (bottom panels) treatment with cetuximab. The tumor lesions are marked with red arrows. The CT scans shown correspond to the pEGFR plasmatic levels marked in panel A with red arrows.

Figure 1

A


Cetuximab
EGF
2D
G12V
G13D
wt
-70
-50
-30
-10
10
30
50
70
% proliferation

B


Cetuximab
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3D
G12V
G13D
wt
-70
-50
-30
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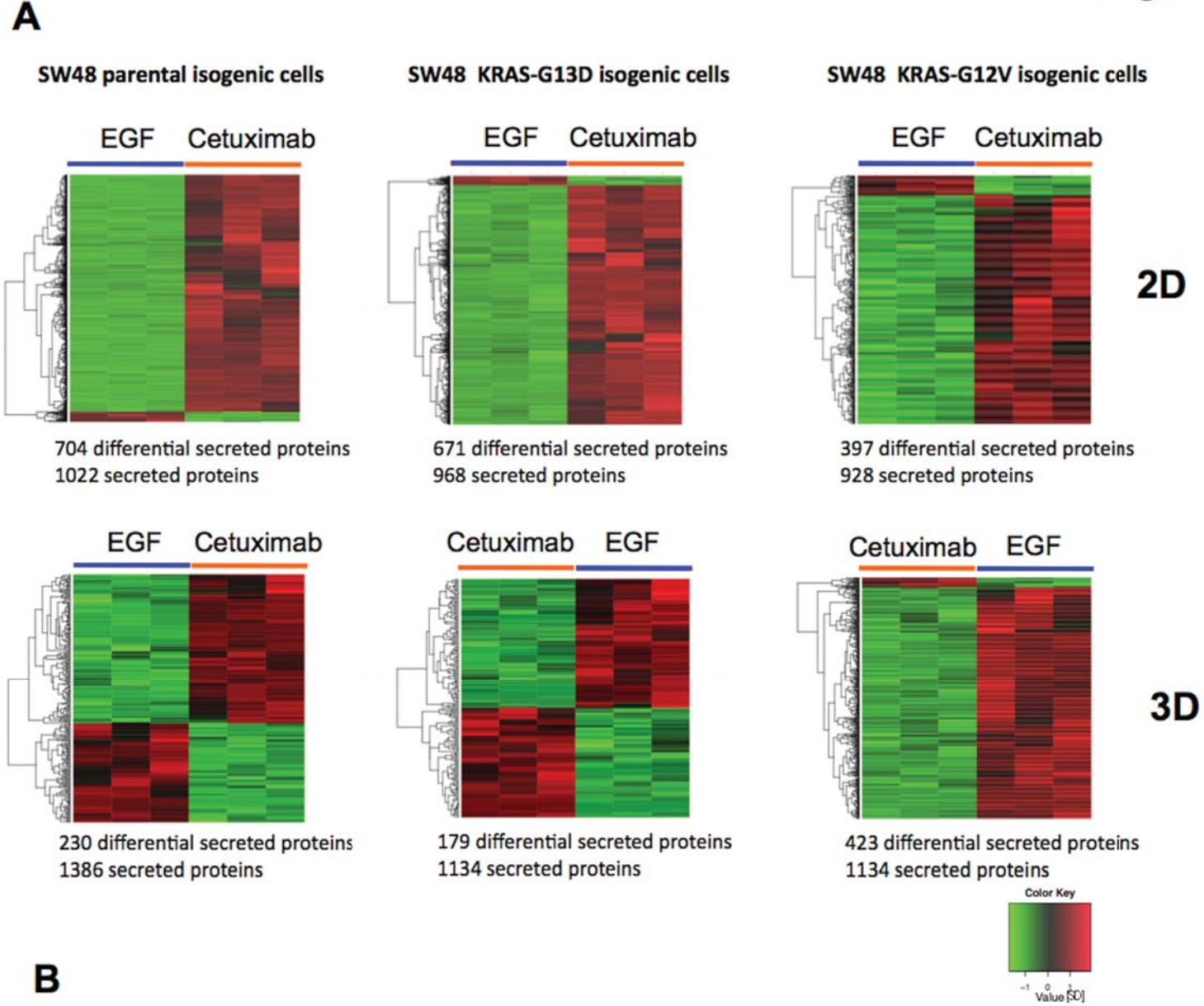
30
50
70
% proliferation


A
SW48 parental isogenic cells
SW48 KRAS-G13D isogenic cells
SW48 KRAS-G12V isogenic cells
EGF
Cetuximab
2D
704 differential secreted proteins
1022 secreted proteins
671 differential secreted proteins
968 secreted proteins
397 differential secreted proteins
928 secreted proteins
Cetuximab
EGF
3D
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1386 secreted proteins
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1134 secreted proteins
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Color Key
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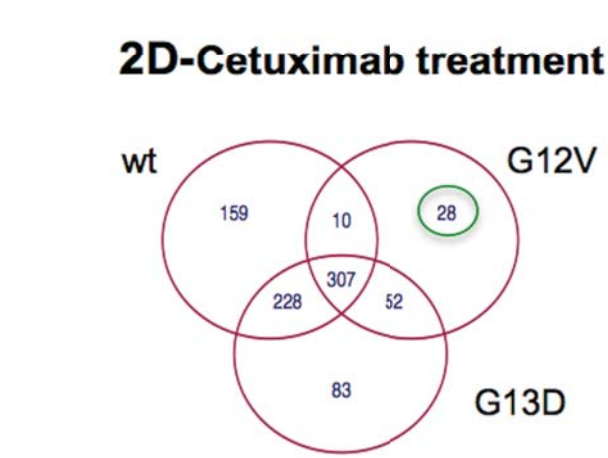
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wt
G12V
G13D
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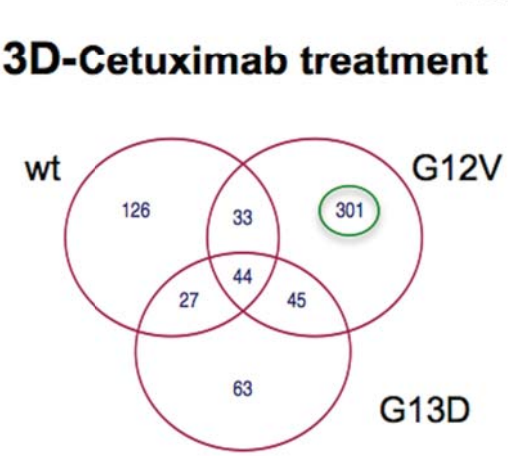
10
28
307
228
52
83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G13D
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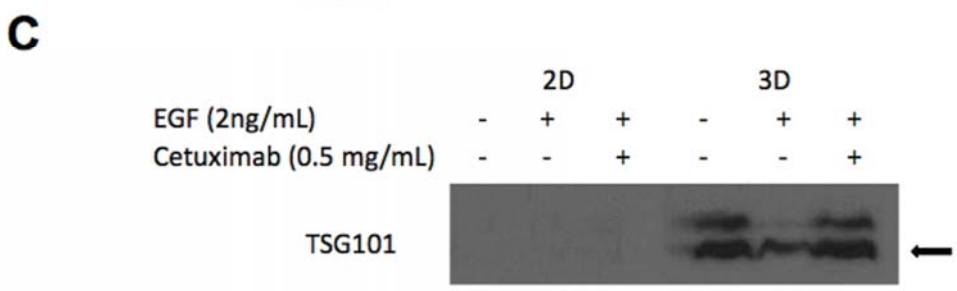
33
301
44
27
45
63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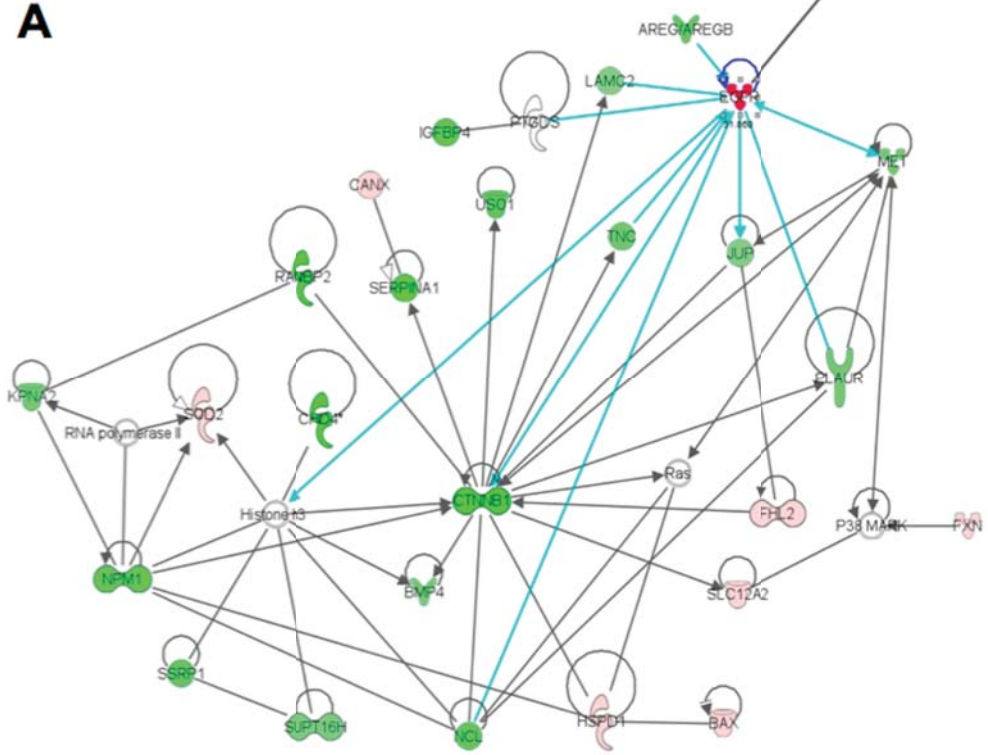
2D
3D
EGF (2ng/mL)
Cetuximab (0.5 mg/mL)
TSG101

Figure 3

A



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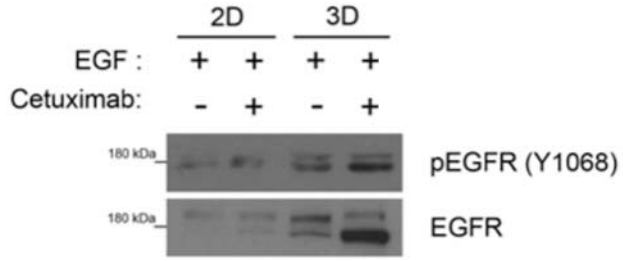

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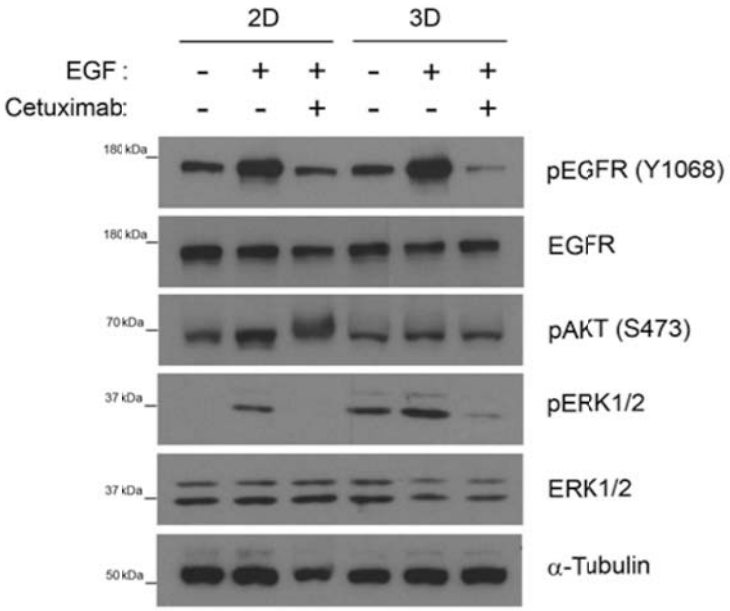

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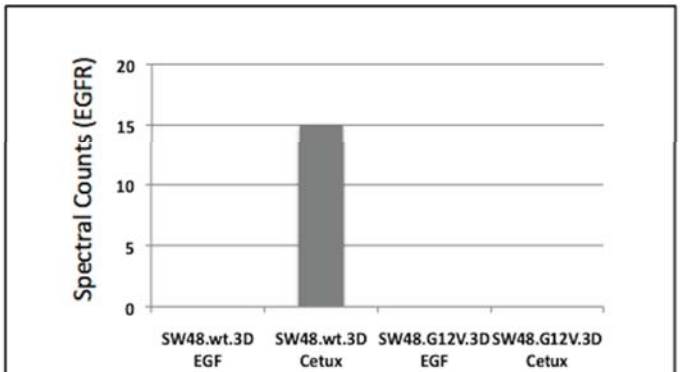
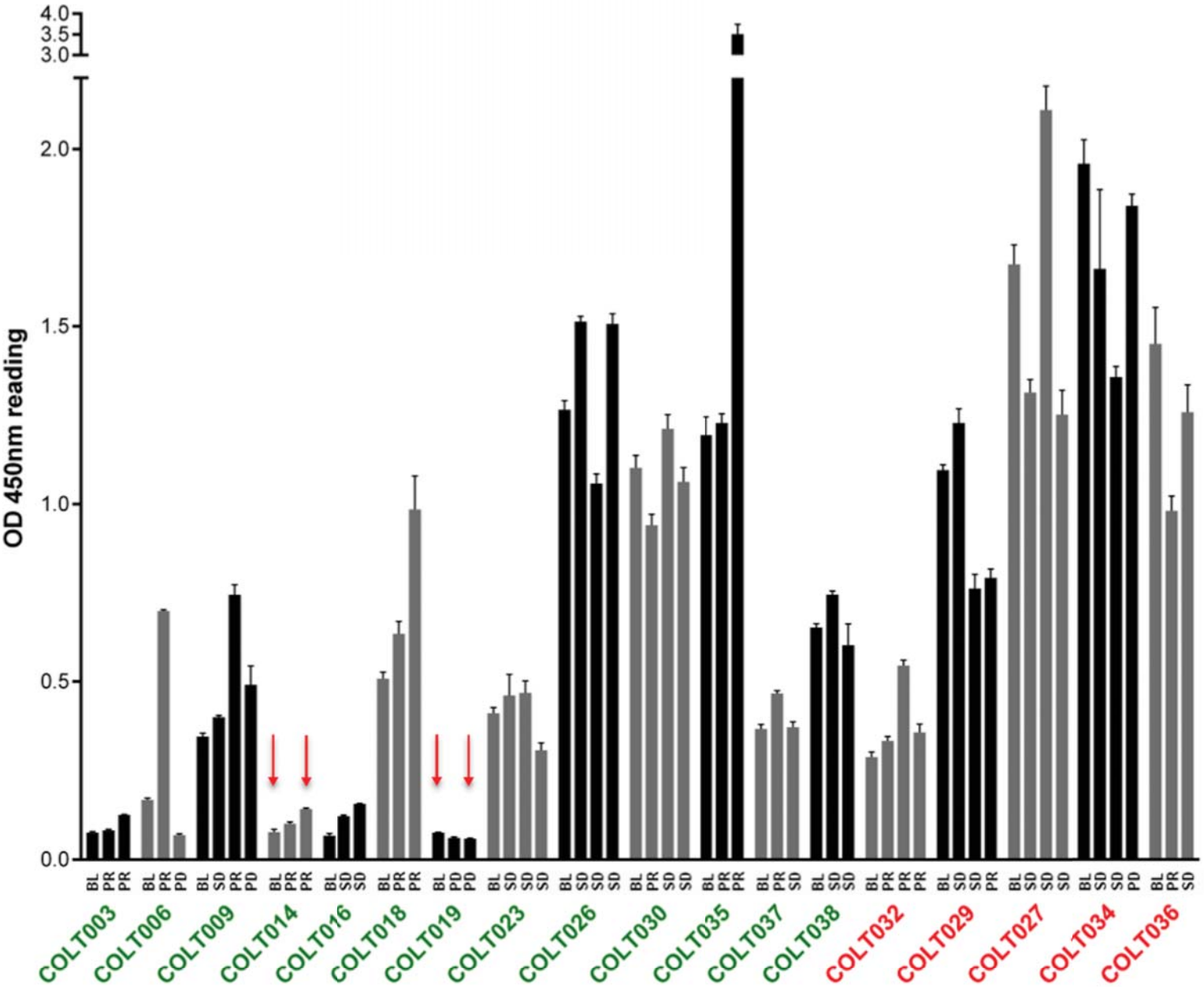


A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BL PR PD
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BL SD SD
BL PR PR
BL PD PD
BL SD SD SD
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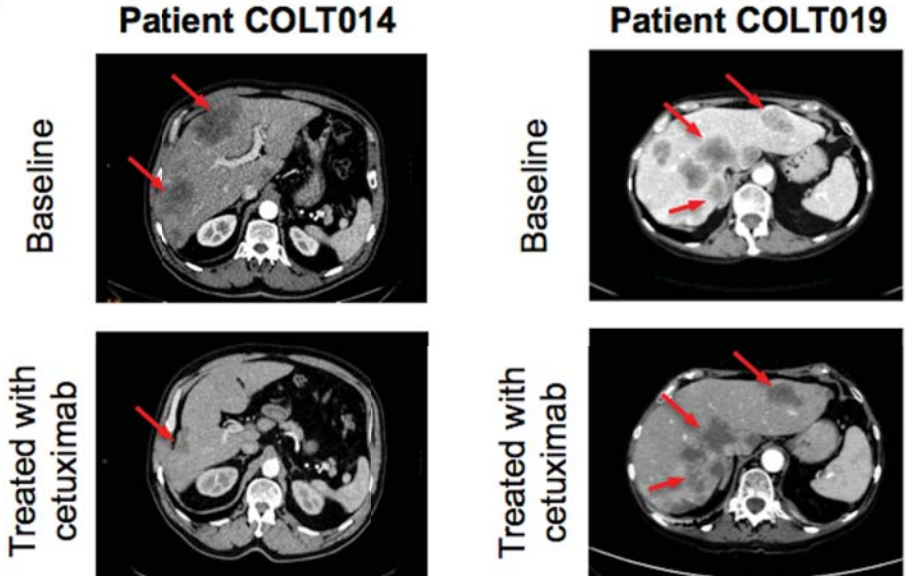
B


Patient COLT014
Patient COLT019
Baseline
Baseline
Treated with cetuximab
Treated with cetuximab