A molecularly annotated platform of patient-derived xenografts ('xenopatients') identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer

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ABSTRACT

Only a fraction of patients with metastatic colorectal cancer (mCRC) have clinical benefit from therapy with anti-EGFR antibodies, which calls for the identification of novel biomarkers for better personalized medicine. We produced large xenograft cohorts from 85 patient-derived, genetically characterized mCRC samples (‘xenopatients’) to discover novel determinants of therapeutic response and new druggable driver oncoproteins. Serially passaged tumors retained the morphological and genomic features of their original counterparts. A validation trial confirmed the robustness of this approach: xenopatients responded to the anti-EGFR antibody cetuximab with rates and extents analogous to those observed in the clinic and could be prospectively stratified as responders or nonresponders based on several predictive biomarkers. Genotype-response correlations indicated HER2 amplification in 2.7% of unselected tumors and in 36% of cetuximab-resistant, KRAS/NRAS/BRAF/PIK3CA wild-type cases. Importantly, HER2 amplification was also enriched (13.6%) in clinically nonresponsive KRAS wild-type patients. A proof-of-concept, multi-arm study in HER2-amplified xenopatients revealed that combined inhibition of HER2 and EGFR induced overt, long-lasting tumor regression. Our results suggest promising therapeutic opportunities in cetuximab-resistant mCRC patients, whose medical treatment in the chemorefractory setting remains an unmet clinical need.

SIGNIFICANCE

Direct transfer xenografts of tumor surgical specimens conserve the interindividual diversity and the genetic heterogeneity typical of the tumors of origin, combining the flexibility of preclinical analysis with the informative value of population-based studies. Our suite of patient-derived xenografts from metastatic colorectal carcinomas reliably mimicked disease response in humans, prospectively recapitulated biomarker-based case stratification, and identified HER2 as a predictor of resistance to anti-EGFR antibodies and as a predictor of response to combinatorial therapies against HER2 and EGFR in this tumor setting.
INTRODUCTION

With a global incidence of over one million cases and a disease-specific mortality of about 33% in the developed world, colorectal cancer (CRC) is a major health burden (1). Despite the introduction of both newer cytotoxic chemotherapies and novel biologic agents, overall progress has been more modest than had been hoped, and metastatic CRC (mCRC) remains largely incurable (2).

As for other types of malignancy, CRC is not a homogeneous disease but actually comprises multiple entities that vary in natural history and molecular pathogenesis. This heterogeneity explains why molecular cancer therapeutics against individual disease driver targets have proven to be effective in only a fraction of cases. One prototypical example is provided by the anti-EGFR monoclonal antibodies cetuximab and panitumumab, which are approved for metastatic colorectal cancer. In unselected patients, the extent of clinical benefit from monotherapy with either drug hovers near the threshold for statistical significance, with response rates of approximately 10% (3-5). The population of potential responders has been recently enriched thanks to a biomarker-development strategy based on the plausible biological rationale that constitutive activation of signaling pathways collateral with or downstream from EGFR, such as the RAS-RAF axis, should circumvent EGFR inhibition and therefore preclude sensitivity to EGFR-targeted agents (6). Indeed, both retrospective and prospective trials have convincingly demonstrated the inefficacy of EGFR neutralizing antibodies in mCRC patients with common (codon 12 and 13) KRAS mutations (7-12). Along this line, a number of retrospective studies have provided initial evidence that rare KRAS mutations as well as NRAS, BRAF and (possibly) PIK3CA mutations are also significantly associated with low response rates (13-16). When considering the cumulative incidence of these genetic alterations in all mCRCs, more than 50% of tumors are expected to be resistant to EGFR targeted antibodies (16). Yet, the overall picture is far from being completed: among the subpopulation that carries wild-type KRAS alleles, objective response to single-agent anti-EGFR treatment is still confined to 13-17% of cases (4,5,17,18).

Although prospective exclusion from treatment of NRAS, BRAF, PIK3CA and rare KRAS mutations will likely lead to a further increase in the percentage of responders, the paucity of therapeutic opportunities remains evident. Patient stratification needs to be refined with further validation of existing biomarkers and with the identification of novel ones; perhaps more importantly, best supportive care remains the only therapeutic option for the large fraction of subjects with cetuximab- or panitumumab-resistant tumors, once they become refractory to standard chemotherapy. We decided to address at least some of these unmet medical needs through a preclinical effort that embodied prospective, randomized trials in mice. To this aim, we took advantage from patient-derived direct transfer xenografts of a large series of liver metastases from CRCs, part of which had been previously deployed for unrelated work (19). We structured the study...
in two consecutive and complementary parts. The first setting was a reverse validation analysis in which existing clinical information was exploited to understand how closely our approach recapitulated the situation in CRC patients. The second setting was an exploratory, proof-of-concept study in which we sought to identify new molecular biomarkers of resistance to anti-EGFR antibodies and test their potential as alternative therapeutic targets. Results from these investigations support using direct transfer xenografts as a reliable strategy to anticipate clinical findings, provide direction for optimizing personalized treatment in mCRCs, and suggest novel treatment opportunities in patients with no other therapeutic options.
RESULTS

Setup and characterization of the xenopatient platform

We undertook a systematic effort aimed to build a biobank of surgical material stored under viable conditions and serially propagated in mouse recipients, starting from CRC liver metastases (19). A summary of the clinical and molecular characteristics for the study population can be found in Table 1 and detailed information is provided in Supplementary Tables S1 and S2. For each tumor specimen, some fragments were ad hoc processed for pathological and molecular characterization; two others were subcutaneously implanted in two different mice and then expanded to generate a pair of independent xenograft lines (‘xenopatients’) from the same patient tumor (Fig. 1A). By combining the use of severely immunocompromised NOD/SCID animals with optimization of patient-to-mouse transfer procedures, we were able to achieve a large percentage of successful engraftments, with 130 out of 150 consecutive specimens (87%) giving rise to transplantable tumors. Therefore, we can reasonably exclude any strong bias towards selection of more aggressive cases in our set of xenografts. It is worth noting that the 20 tumors that failed to engraft tended not to harbor mutations in KRAS, NRAS, BRAF, and PIK3CA: specifically, of the 14 samples for which genetic information was available, only two proved to be mutated (one for KRAS and another for PIK3CA; Supplementary Table S2). While this finding might indicate a potential association between engraftment efficiency and mutational status, the numerousness and high take rate of implanted tumors allowed a representation of wild-type and mutated cases at frequencies similar to those described in other series of metastatic CRCs (16).

To rule out any major phenotypic drift that xenografted specimens might have acquired due to sequential passaging, we compared the histological aspects of the tumors grown in mice (explanted after the second in vivo passage) with those of the corresponding original metastasis in 22 randomly selected cases. In line with previous findings (20), we observed substantial preservation of morphological traits between patient-derived and mouse-derived tumors (Fig. 1B).

Mouse-passaged CRC samples have already been used for genome-wide mutational studies and for copy number variation surveys (21,22). While genetic coherence between fresh and mouse-expanded material has been demonstrated in the case of missense mutations (23), a systematic comparative assessment of gene copy number changes has not been attempted so far. We therefore used SNP arrays to obtain an unbiased copy number assessment of 39 tumors derived from four different patients. For each patient, we analyzed the DNA derived from different sources: i) normal liver; ii) original liver metastasis; iii) mouse-grown tumors explanted after the first passage in vivo (one or two samples per patient); iv) mouse-grown tumors explanted after the second passage in vivo (four or eight samples per patient). We detected largely concordant copy number variations between first- and second-passage xenografts and their matched original counterparts,
whereas a marked heterogeneity was observed among tumors belonging to different individuals (Fig. 1C and Supplementary Fig. S1). For some loci, genomic consistency was higher among propagated samples than between passaged and fresh specimens (Supplementary Fig. S1). This is likely due to substitution of human stroma with host murine components during serial expansion in vivo; because the SNP probes are human-specific, dilution of tumor genomic content by DNA from the normal human microenvironment occurs in original samples but not in xenografted tumors. The same set of fresh and passaged specimens was also profiled for KRAS, NRAS, BRAF, and PIK3CA mutations in multiple hotspots: genetic lesions detected in the original samples were maintained in the xenografted tumors (which did not display further mutations in the examined genes) and wild-type cases persisted unaltered throughout serial passaging (Supplementary Table S2). Overall, these results reinforce the notion that tumors largely retain their phenotypic and genomic characteristics during early passages in mice and substantiate the potential of patient-derived xenografts as faithful aliases of human patients for preclinical studies.

Design of reverse validation trial with cetuximab

The trial was performed on 85 mCRC samples that had successfully engrafted. Of these, two (2.3%) displayed high-grade microsatellite instability (Supplementary Table S2), consistent with the 1.3-2.7% frequency already described in metastatic colorectal cancer (13,24). Xenografts were propagated through double-step in vivo passaging and treatment cohorts of 12 mice were generated from each implanted specimen. In 57 cases (67%) we obtained productive development of two independent cohorts from two different fragments of the same original tumor; for the remaining 28 samples (33%) engraftment occurred in just one animal, and only one cohort was obtained. When tumors in each cohort reached an average volume of 400 mm$^3$, mice were randomized to receive either placebo or cetuximab (Fig. 1A). Based on this experimental pipeline, we denominate ‘case’ the average performance of one or (more frequently) two lines of xenopatients from one single patient.

For assessment of tumor response to therapy, we used volume measurements and adopted a classification methodology loosely inspired to clinical criteria: i) tumor regression (or shrinkage) was defined as a decrease of at least 50% in the volume of target lesions, taking as reference the baseline tumor volume; ii) at least a 35% increase in tumor volume identified disease progression; and finally, iii) responses that were neither sufficient reduction to qualify for shrinkage nor sufficient increase to qualify for progression were considered as disease stabilization.

We designed the experiments following a historical perspective. In a first phase, the trial was performed on an initial unselected population of 47 cases. All cohorts were treated with cetuximab or placebo independent of KRAS mutational status, to analyze whether KRAS mutant xenopatients were in fact resistant to anti-EGFR treatment and to compare our response rates with those from...
the first trials in the pre-KRAS selection era. In the second phase, KRAS mutant tumors were spared from treatment, and cetuximab was given to 38 newly accrued cases that exhibited wild-type KRAS (and included, however, all other mutant genotypes). Hence, this second part of the trial recapitulated the current guidelines for EGFR-targeted therapy in mCRCs.

**Effect of cetuximab treatment in patient-derived mCRC xenografts and biomarker analysis**

A first evaluation was scheduled three weeks after treatment initiation. In an unselected population of 47 mCRCs, we observed tumor shrinkage in five cases (10.6%), disease stabilization in 14 cases (29.8%) and progression in 28 cases (59.6%) (Fig. 2A and B and Supplementary Table S2). Tumors displaying regression or stabilization continued treatment for additional three weeks. At this second timepoint, shrinkage was confirmed in five cases and was monitored in two other cases that had experienced prior stabilization; stable disease was maintained in eight cases and turned into progression in four cases (Supplementary Fig. S2). Collectively, these response rates are coherent with those obtained in unselected patients treated with single-agent cetuximab or panitumumab. By performing a meta-analysis of the available data on the activity of anti-EGFR monotherapy in both heavily pretreated and chemo-naïve subjects, regardless of KRAS status, we found that average objective response was 10% (range 8-11.6%) and stable disease occurred in 30% of cases (range 24-37%) (4,5,25-29).

When reconsidering response rates according to the mutational status of KRAS, neither regression nor disease stabilization was achieved in any of the 18 cohorts bearing KRAS (codons 12 and 13) mutant tumors (38.3%) (Fig. 2A, Supplementary Fig. S2, and Supplementary Table S2), again in agreement with clinical evidence (7-12). Two KRAS-mutant cases displayed stable disease at the first three-week evaluation timepoint, but they underwent progression in the following three weeks (Supplementary Fig. S2).

Having confirmed that KRAS mutant mCRCs were resistant to cetuximab in this first series of consecutive samples, we elected to exclude from treatment all additional cases with codon 12 and 13 mutations. Fig. 3A displays the effect of cetuximab administration in the overall KRAS (codon 12 and 13) wild-type population after three weeks; the plot comprises a total of 66 wild-type tumors, 28 from the first series and 38 collected thereafter. In this patient subgroup, tumor regression increased to 16.7% (11 cases), disease stabilization rate was 40.9% (27 cases), and tumor progression rate was 42.4% (28 cases) (Fig. 3A and Supplementary Table S2). At the six-week timepoint, disease stabilization changed to tumor shrinkage in three cases and developed into disease progression in seven cases (Supplementary Fig. S3 and Supplementary Table S2). Once more, these distributions are in agreement with clinical studies in chemotherapy-refractory mCRC patients, which describe a response rate of 12.8-17% and disease stabilization in 34% of KRAS (codon 12 and 13) wild-type patients (17,18).
A number of retrospective studies have suggested that mutations in other downstream effectors of the EGFR signaling pathway, such as NRAS, BRAF and PIK3CA, might also have a negative effect on response to anti-EGFR antibodies (13-16). Since our KRAS (codon 12 and 13) wild-type subpopulation included all these genotypes and additional KRAS rare mutations at codons 61 and 146, we decided to exploit our platform to challenge the predictive value of these emerging biomarkers. Our results prospectively validated observations in patients: none of the seven NRAS- and three BRAF-mutant cases (10.6% and 4.5%, respectively) or the four cases with KRAS rare mutations (6.1%) responded to cetuximab with tumor shrinkage or stabilization.

Four tumors (6.1%) harbored a PIK3CA mutation without concurrent KRAS mutations; of these, three (one exon 9 and two exon 20 mutants) progressed, and one (exon 20) regressed (Fig. 3A, Supplementary Fig. S3, and Supplementary Table S2). These results are partially at odds with previous reports suggesting that mutations in exon 20, but not exon 9, predict worse outcome after cetuximab (14-16). However, due to the low number of patients both in our series and in the previous studies, the impact of this potential ‘exon-specific’ effect remains uncertain. When restricting the analysis to the ‘quadruple negative’ subset exhibiting wild-type KRAS, NRAS, BRAF and PIK3CA, the percentage of responders was enriched up to 20.8% and disease stabilization rose to 56.3%.

We further assessed the predictive power of additional biomarkers that have been proposed as positive determinants of sensitivity to cetuximab. An increase in EGFR gene copy number, usually due to a variable degree of chromosome 7 polysomy rather than locus-specific amplification, has been found to correlate with response to anti-EGFR therapies (30-33). In KRAS wild-type tumors, it has been demonstrated that patients with an EGFR FISH-positive phenotype show higher response rates (71%) compared with patients with normal EGFR copy number (37%) (34). In our series, EGFR copy number gain (as assessed by qPCR) was detected in 23 out of 66 KRAS (codon 12 and 13) wild-type cases (34.8%) and was associated with regression in seven cases (30.4%), stabilization in 12 cases (52.2%), and progression in four cases (17.4%). The 43 tumors with normal EGFR copy number (65.2%) displayed shrinkage in four cases (9.3%), stabilization in 15 cases (34.9%) and progression in 24 cases (55.8%) (Fig. 3B and Supplementary Table S2). The frequency of disease stabilization in tumors with EGFR copy number gain was even higher when considering only the ‘quadruple negative’ subpopulation (Fig. 3B). Therefore, EGFR copy number gain tended to segregate responders also in our preclinical context.

Preliminary evidence from small series and then larger retrospective studies have shown an association between expression of amphiregulin and epiregulin, two EGFR ligands, and clinical outcome (35,36). In patients with wild-type KRAS tumors, a significant association was observed between ligand expression and tumor shrinkage or stabilization; however, objective responses were also detected in patients with low ligand expression (36). Again, our preclinical trial provided
analogous information. By performing qRT-PCR on the original material of 54 KRAS wild-type tumors, a trend was noticed towards higher median expression of both amphiregulin and epiregulin in responders, but cases of tumor regression also occurred in tumors with low ligand expression (Fig. 3C and Supplementary Table S2).

**Correlation of HER2 amplification with therapeutic resistance to cetuximab**

Despite the enrichment in responders and the higher frequency of stable disease compared to the KRAS wild-type (codons 12 and 13) subpopulation, 11 out of 48 cases (22.9%) with quadruple negative tumors proved to be resistant to cetuximab (Fig. 3A). Similar resistance rates for the same genetically defined subgroup have been described in retrospective clinical studies (16). This calls for the identification of other molecular determinants responsible for de novo therapeutic resistance within this patient subset. This task is further supported by the notion that resistance biomarkers, if druggable, can represent alternative therapeutic targets per se.

Overexpression of receptor tyrosine kinases (RTKs) other than EGFR has been shown to obviate for need of activated EGFR signaling and be responsible for resistance to anti-EGFR therapies in various tumor settings (37). We therefore concentrated on RTKs known to be deregulated in colorectal cancer. Genome-wide expression profiling in a suite of 137 mCRCs that included most of the KRAS wild-type tumors used in our reverse validation trial with cetuximab (51 out of 66, 77%) indicated high-level expression of a number of RTKs in individual samples. Among these, HER2 stood out from the survey due to the presence of three outliers featuring massive receptor overexpression (2.2%) (Fig. 4A). This prevalence is in agreement with other population studies, which report HER2 overexpression in 2-3.5% of genetically unselected CRCs as a consequence of locus-specific gene amplification (38-40). We confirmed this prevalence in a second independent case series: tissue microarray-based analysis of 112 mCRC archival specimens revealed concordant HercepTest and FISH positivity for HER2 overexpression/amplification in three cases (2.7%) (Fig. 4B).

When performing genotype-response correlations, we found interestingly that the three HER2-overexpressing outliers pinpointed by genome-wide transcriptional analysis (M051, M077, and M091) all fell within the quadruple negative subset and all displayed unquestionable resistance to cetuximab (Supplementary Fig. S4). The collection used for gene expression profiling contained only seven of the 11 cetuximab-resistant cases with a quadruple negative genotype present in our set of KRAS wild-type xenopatients. To extend our survey and to support expression data with genetic information, we performed qPCR gene copy number analysis on the whole panel of cetuximab-resistant, quadruple negative cases. Genomic amplification of HER2 was confirmed in the three expression outliers previously identified by gene expression analysis and was found in a fourth case (M147) (Fig. 4C and Supplementary Fig. S4); results were finally validated by FISH (Fig.
Therefore, the prevalence of HER2 amplification rose from 2-3% to more than 36% (4/11) when considering cases resistant to cetuximab that were wild-type for KRAS, NRAS, BRAF and PIK3CA (Fig. 4E).

To establish the clinical relevance of these results, we explored whether HER2 was overexpressed and/or amplified in tumors from patients that exhibited resistance to anti-EGFR antibodies. We started with a retrospective analysis of cases collected at our Institutions. In 17 KRAS wild-type mCRCs from patients that had not received clinical benefit from either cetuximab or panitumumab (16 progressive diseases and one ephemeral stabilization that shortly turned into progressive disease), we found three cases with a positive (3+) HercepTest and diffuse, high-grade amplification of HER2 (3/17, 17.6%) (Fig. 5A and Supplementary Table S3). In contrast, no HercepTest-3+ overexpression or homogeneous amplification of HER2 could be detected in any of 14 KRAS wild-type tumors from patients in which anti-EGFR treatment had induced disease control (nine objective responses and five long-lasting stable diseases) (Supplementary Table S3). Then, we searched for HER2-overexpressing outliers using publicly available gene expression datasets annotated for response to anti-EGFR antibodies. The first database comprises 59 colorectal carcinomas, including 39 KRAS wild-type cases; among this latter group, 20 tumors are reported to be cetuximab-sensitive (objective response or stable disease) and 19 are defined as cetuximab-resistant (progressive disease) (35); a second independent database encompasses 19 KRAS wild-type tumors, of which 11 are annotated as cetuximab-sensitive and eight as cetuximab-resistant (41). Results from this analysis were consistent with data from our internal series. In the first collection, two HER2 overexpressors could be identified (Supplementary Fig. S5): both were wild-type for KRAS and segregated in the subset of cetuximab-resistant tumors (2/19, 10.5%) (35). The second dataset reported the presence of one case featuring HER2 overexpression, which was categorized as a nonresponder (1/8, 12.5%) (41). Collectively, these figures indicate a frequency of HER2 amplification/overexpression in 13.6% of patients with KRAS wild-type tumors that progressed on cetuximab or panitumumab (6/44) (Fig. 5B), with no evidence of HER2 amplification in any of the 45 patients in which anti-EGFR therapy was effective ($p < 0.05$ by Fisher’s exact test).

Although limited in number, these findings strongly suggest that HER2 amplification is a negative determinant of response to anti-EGFR antibodies in mCRCs that do not harbor genetic alterations of the RAS pathway.

**Therapeutic opportunities for cetuximab-resistant, HER2-amplified colorectal tumors**

We reasoned that HER2 amplification could represent not only a biomarker of resistance to EGFR inhibition but also a positive predictor of response to HER2-targeting agents. Furthermore, HER2 inhibition could restore sensitivity to anti-EGFR therapies. To assess the validity of HER2 as a therapeutic target in cetuximab-resistant mCRCs, we exploited the experimental merits of our
xenopatient platform by executing a proof-of-principle, multi-arm ‘xenotrial’ in two representative cases (M077 and M091). As for the choice of therapeutic regimens, we focused on dual inhibitors of HER2 and EGFR that were administered individually, together, or in combination with cetuximab. We selected pertuzumab, a recombinant humanized monoclonal antibody that disrupts HER2 heterodimerization with EGFR and with other HER partners (42), and lapatinib, a small molecule inhibitor with high selectivity for both HER2 and EGFR (43). For each case, the original tumor specimen was serially passaged in vivo until production of 30 tumor-bearing animals. When xenografts reached an average volume of approximately 400 mm$^3$, mice were randomized into six independent treatment cohorts, each consisting of five xenopatients: i) vehicle (placebo); ii) pertuzumab alone; iii) pertuzumab and cetuximab; iv) lapatinib alone; v) lapatinib and cetuximab; vi) lapatinib and pertuzumab.

At the first evaluation timepoint after three weeks of treatment, case M077 proved to be completely insensitive to pertuzumab alone (tumor volume variation compared with volume at baseline: +196%). The combination of pertuzumab and cetuximab produced only a negligible growth delay (+123%), after which mice needed to be sacrificed. Lapatinib alone displayed stronger activity and induced disease stabilization (+10%). Intriguingly, the association of lapatinib and pertuzumab or lapatinib and cetuximab resulted in important tumor volume reduction, with the former combination being more effective (-75% for pertuzumab:lapatinib and -41% for cetuximab:lapatinib) (Fig. 6A). Tumor regression in all arms treated with antibody and lapatinib became massive in the second three-week observation period (-93% for pertuzumab:lapatinib and -79% for cetuximab:lapatinib), whilst disease stabilization produced by lapatinib alone tended to attenuate over time (+37%) (Fig. 6A). Of note, delayed addition of lapatinib in a treatment arm that had received only pertuzumab for the first four weeks rendered pertuzumab-insensitive, exponentially growing tumors fully responsive to the combination therapy, with rapid and dramatic tumor shrinkage (Supplementary Fig. S6A). Response of case M091 to the same treatment modalities was similar, with some minor differences. In line with the results obtained in M077, pertuzumab alone was completely ineffective (+237% after three weeks of therapy), whereas the combination of pertuzumab and lapatinib led to overt tumor regression (-65% after three weeks and -64% after six weeks). Again in accordance with findings in case M077, the effect of cetuximab:lapatinib was considerable, but less pronounced than that exerted by pertuzumab:lapatinib (-53% after three weeks and -45% after six weeks). Different from M077, disease stabilization was induced by double therapy with pertuzumab and cetuximab (-11% after three weeks and +15% after six weeks) but not by lapatinib alone (+163% after three weeks) (Fig. 6B).
In situ examination of representative tumors from case M077 using phospho-specific antibodies directed against HER2 and EGFR downstream transducers revealed that treatments were efficacious only when they fully neutralized signal activity. Phosphorylation of the MEK substrate ERK and of the PI3K distal effector S6 was impaired very weakly by pertuzumab alone or by pertuzumab:cetuximab (Supplementary Fig. S6B) and only partially by lapatinib (Fig. 6C); conversely, treatment with lapatinib:pertuzumab or lapatinib:cetuximab resulted in complete signal abrogation in cancer cells (Fig. 6C). At the morphological level, the lingering tumor tissue retrieved after prolonged treatment with lapatinib and antibody consisted of residual pseudoglandular islands lined by pluristratified epithelium and embedded in large necrotic areas (Fig. 6C). Together, these results provide strong indication that the association of a dual EGFR/HER2 small molecule inhibitor and a monoclonal antibody directed against either EGFR or the EGFR/HER2 heterodimer might prove beneficial to treat cetuximab-resistant, quadruple negative, HER2-amplified mCRCs in the clinical setting.
DISCUSSION

Preclinical validation of potential therapeutic targets using in vivo models is traditionally regarded as an obligatory step of anticancer drug development but it is also considered a problematic issue. There is now rising concern that what is still deemed a successful endpoint at the preclinical level – positive performance of a drug in xenografts of different human cancer cell lines – is in fact not predictive of compound efficacy in the clinical setting (44). The obvious objection is that immortalized cancer cells, which are commonly employed in xenograft experiments, have been adapted to grow on plastic in the laboratory for decades and thus exhibit a genetic drift, a biological compliance and phenotypic features different from original cancers in patients. Besides this evident flaw, another (often underestimated) drawback of such an approach is that the catalog of currently available cell lines is inevitably finite, and possibly poor for some tumor types. The main reason for this inadequacy is an inherent difficulty in deriving long-term cell cultures from human tumors, which not only limits the spectrum of accessible cellular models but also introduces a heavy bias for selection of aggressive subtypes, which are likely more amenable to in vitro propagation. This restricted compendium of representative ‘cases’ is in conflict with the notion that each cancer in each individual is a separate entity, endowed with a unique natural history and riddled with a number of unpredictable patient-specific interacting events. Therefore, experiments with cell lines cannot recapitulate the wide heterogeneity of human malignancy that occurs among individuals on a population basis. The absence of genetic diversity, or at least a strong tendency towards an artificially uniform tumor evolution, is also a pitfall of genetically defined mouse models of cancer, which usually develop stereotypical lesions triggered by the same initiating oncogenic hit (45).

One robust way to proceed with efficient, high-fidelity drug development at the stage of in vivo validation, while minimizing the effects of uncharacterized tumor heterogeneity, would be to perform preclinical population-based studies. To do this, we reasoned that a practicable model was the use of a series of human cancer specimens directly transplanted into mice, in order to generate a study population that could be concomitantly profiled for biomarker assessment and randomized for prospective treatment with targeted agents (‘xenopatients’). By optimizing some procedural tips, we were able to achieve high rates of successful sample engraftments in mice, thus ruling out a nonrandom prevalence of aggressive tumors. We then afforded the issue of model predictivity analytically, using a reiterative strategy; in particular, we exploited the molecular and clinical knowledge accumulated on targeted therapy with the anti-EGFR antibody cetuximab, in terms of response rates and biomarkers, and challenged the predictive value of the xenotransplantation setting by investigating whether what had been demonstrated in humans also applied to mice.

Several lines of evidence support the robustness and the predictive power of our strategy: i) Notwithstanding their ectopic (subcutaneous) site of growth, mCRC xenografts retained the...
morphological characteristics of the corresponding patient’s lesion, with aspects of glandular differentiation, mucinous histology or anaplastic pleomorphism that were in agreement with the original phenotypes. ii) A genome-wide survey of SNP variations and a more oriented mutational analysis, both performed on a representative series of matched primary and xenografted cases, revealed that serial mouse passaging did not grossly alter the genetic makeup of tumors, at least when considering global copy number changes and hotspot oncogenic mutations. iii) In the majority of cases, we monitored concordant effects of cetuximab in parallel xenograft cohorts derived from different specimens of the same tumor. This suggests that there was no critical sampling bias in the random selection of cancer fragments for implantation and that intrinsic genetic heterogeneity or regional clonal discrepancies did not influence the overall tumor sensitivity to therapy. iv) The frequency of tumor regression, disease stabilization and disease progression following cetuximab treatment was in line with the clinical data reported in humans; and, identical to clinical observations, KRAS (codon 12 and 13) mutant xenografts were all resistant to EGFR blockade.

Consistency between patients and mice was also noticed for those potential biomarkers that have weaker significance or await further validation. In accordance with clinical information, a grey zone of inconclusive findings emerged when we evaluated proposed positive response determinants, namely, EGFR gene copy number gain and overexpression of the EGFR ligands amphiregulin and epiregulin (30-36). A trend was observed whereby these parameters appeared to be enriched in responders, but the absence of unambiguous cutoff criteria confirmed that these biomarkers remain unfit for reliable case stratification. Instead, results were straightforward when we analyzed candidate predictors of lack of response, in particular KRAS rare mutations, NRAS and BRAF mutations (13-16): such genotypes all associated with resistance to cetuximab. By confirming in a prospective manner previous retrospective clinical data, our findings advocate inclusion of these negative biomarkers in the toolbox of surgical pathologists.

Our observation that HER2 is amplified in a small percentage (2-3%) of genetically unselected CRCs is in agreement with previous reports (38-40). The unexpected and novel finding is the higher frequency of HER2 amplification in cetuximab-resistant cases and its progressive enrichment along with refinement of genetic selection. Indeed, in KRAS wild-type CRC patients that displayed de novo resistance to anti-EGFR therapies, high-grade HER2 amplification was detected in six out of 44 cases (13.6%). Further, in a more defined subset of KRAS/NRAS/BRAF/PIK3CA wild-type xenopatients in which treatment with cetuximab was ineffective, HER2 amplification was detected in four out of 11 cases, with an increase in prevalence up to 36%. Although absolute numbers remain small, it appears clear that the cases of HER2 amplification all segregated with lack of response to anti-EGFR therapy, both in xenopatients and – importantly – in patients. At the genetic level, the role of HER2 as a potential driver of therapeutic resistance is supported by the mutual exclusivity between HER2 amplification and mutations in components of substitute signaling pathways,
including KRAS, NRAS, BRAF and PIK3CA, which are also molecular determinants of resistance. Larger scale retrospective trials are warranted to strengthen the role of HER2 as a resistance biomarker and prospective studies are necessary for ultimate validation in patients. Given the low frequency of HER2 amplification in unselected populations, critical logistical aspects that trial designers will have to deal with are optimization of patient accrual and implementation of multi-parametric genetic profiling to enrich for patients with quadruple negative tumors.

The frequency of HER2-amplified cases in CRC is similar to that of other proposed biomarkers of resistance, including BRAF or NRAS (16). While no approved therapies currently exist to effectively target BRAF/NRAS mutant tumors, our results indicate that HER2 amplification is not only a negative biomarker of resistance to cetuximab but also a positive biomarker of response to clinically used anti-HER2 therapies. Indeed, we found that dual targeting of HER2 and EGFR induced tumor regression when using an association of lapatinib and pertuzumab or, to a lesser extent, lapatinib and cetuximab. Based on the reliability of xenopatients in mimicking the human situation, we believe that our preclinical findings in HER2-amplified tumors can be considered as a reliable proxy of future findings in patients. In particular, the evident tumor shrinkage produced by the lapatinib:pertuzumab combination bodes well for prospective application of this therapeutic regimen in Phase II clinical trials, of which we strongly encourage timely implementation.

In conclusion, we executed molecularly defined prospective trials in mice for treatment efficacy studies in metastatic colorectal cancer, with the major objective of advancing therapeutic strategies in a setting that best mimics the clinical context in patients. Our preclinical platform provides an instructive framework for additional biomarker discovery, for the generation of predictive classifiers for better patient stratification, and for testing novel investigational therapies that will undoubtedly improve the figures emerged from this initial effort.
METHODS

Specimen collection and annotation
A total of 150 consecutive tumor samples and matched normal samples were obtained from patients treated by liver metastasectomy at IRCC and Mauriziano Umberto I (Torino, Italy). All patients provided informed consent and samples were procured and the study was conducted under the approval of the Review Boards of the Institutions. Clinical and pathologic data were entered and maintained in our prospective database.

Molecular and bioinformatic analyses
Analyte extraction, gene copy number and expression analysis and mutational profiling were performed as described (13, 19, 46). Part of data on EGFR copy number has already been published (19). Primers for HER2 gene copy number analysis were the following: forward, 5'-GTGAGTGATGGGGCTGAGTT-3'; reverse, 5'-CCAGGGAGGAGTGAGTTGTC-3'. Microsatellite instability was analyzed using the MSI Analysis System (Promega). Cases featuring two or more mutant alleles were categorized as microsatellite unstable-high according to the revised Bethesda guidelines (47).

The samples gathered for the copy-number survey of four independent patients (38 tumors and four matched normal tissues) were handled and hybridized as previously described for Affymetrix Human 6.0 SNP array profiles (48). For detecting copy number variations, the Affymetrix Genotyping Console (GTC) 3.0.2 and the Birdseed (v2) algorithm were used. The default external reference provided by the GTC platform was exploited as background. The results from the GTC analysis pipeline were assembled in a data matrix in which a numerical integer value ranging from 0 to 4 was assigned to each of the copy number probes (908226) present in the array. Scores of 0 and 1 were considered as calls for losses, values of 3 and 4 were defined as calls for copy number gains. This matrix was used for subsequent analyses. Hierarchical clustering was performed on the copy number call matrix previously described, using Pearson correlation metrics and complete linkage to reveal similar clusters. All the computations were performed in the R Statistical Environment. To generate the frequency maps, each chromosome was parsed using a sliding window covering 1600 copy-number probes and a sampling step of 800 probes. For each step, we evaluated the frequency of the most prevalent putative copy-number call according to the results obtained with the GTC software. The results were plotted as a heat-map using the gedas software (49).
**Explant xenograft models and in vivo treatments**

Tumor implantation and expansion was performed as previously described (19). Established tumors (average volume 400 mm$^3$) were treated with the following regimens, either single-agent or in combination: cetuximab (Merck) 20 mg/kg, twice-weekly; pertuzumab (Roche Genentech) 20 mg/kg, once-weekly; lapatinib (Sequoia Research Products) 100 mg/kg, daily. Tumor size was evaluated once-weekly by caliper measurements and the approximate volume of the mass was calculated using the formula $4/3\pi(d/2)^2D/2$, where $d$ is the minor tumor axis and $D$ is the major tumor axis. All animal procedures were approved by the Ethical Commission of IRCC and by the Italian Ministry of Health.

**In situ morphological analyses**

Immunohistochemistry was performed as described (50). Images were captured with the Image-Pro Plus 6.2 software (Media Cybernetics) using a BX60 Olympus microscope. Herceptest-based HER2 scoring followed the consensus panel recommendations for gastric cancer (51). FISH analysis was performed as described (52). For automated acquisition, the motorized Metafer Scanning System (Carl Zeiss MetaSystems GmbH) and AxioImager epifluorescence microscope (one focus plane for DAPI and 13 focus planes for green and red spots) were used. Analysis of HER2/CEP17 (chromosome 17 centromere) probes was performed automatically by Metafer through the PathVysion V2 software (FDA-approved).

**Statistics**

Statistical analyses were performed by the two-tailed Student's $t$ test, Chi-square test, Fisher's exact test and binomial distribution calculations using Excel or the R statistical environment. For all tests, the level of statistical significance was set at $p < 0.05$. 


ACKNOWLEDGMENTS

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REFERENCES


LEGENDS TO FIGURES

Figure 1. Setup and characterization of the xenopatient platform
A, Generation of xenopatients. Following surgical removal from patient, each mCRC specimen was cut in small pieces and two fragments were implanted in two mice. After engraftment and tumor mass formation, the tumors were passaged and expanded for two generations until production of two cohorts, each consisting of 12 mice. These were randomized for treatment with placebo (six mice) or cetuximab (six mice). B, Xenografted tumors retained the histopathological characteristics of original samples. H&E stains of representative cases with different morphological features. In some instances, both fresh and passaged lesions displayed a well differentiated phenotype, with cells describing irregular pluristratified tubular/acinar structures with multiple lumens embedded in a scarce stromal matrix. Other samples had a clear-cell appearance or featured high nuclear grade and areas of necrosis. In some cases, discohesive mucus-secreting cells defined a moderately differentiated phenotype typical of mucinous adenocarcinoma, with signet-ring elements showing peripheral nuclear delocalization and abundant intervening stroma associated with desmoplastic reaction. Finally, a few tumors exhibited high-grade pleomorphism and could be pathologically classified as poorly differentiated adenocarcinomas. Bar, 50 μm. C, Genetic concordance between xenografts and their original counterparts. Similar groups of samples are evidenced by applying a Pearson-based hierarchical clustering to copy number calls (see Methods for details).

Figure 2. Effect of cetuximab treatment in unselected mCRC xenopatients
A, Waterfall plot of cetuximab response after three weeks of treatment, compared with tumor volume at baseline, in an unselected population of 47 cases. Dotted lines indicate the cut-off values for arbitrarily defined categories of therapy response: cases experiencing disease progression, stabilization or regression are shaded in light brown, light yellow and light aquamarine, respectively. Asterisks denote the samples for which growth curves are shown in panel B. B, Representative tumor growth curves in cohorts derived from individual patients, treated with placebo (grey) or cetuximab (red). n = 6 for each treatment arm. R, regression/shrinkage; S, stabilization/disease control; S/P, initial stabilization followed by tumor progression; P, progression/lack of response.

Figure 3. Effect of cetuximab treatment in KRAS wild-type mCRC xenopatients and biomarker correlations
A, Waterfall plot of cetuximab response after three weeks of treatment, compared with tumor volume at baseline, in a KRAS wild-type population of 66 cases. B, Distribution of response rates based on EGFR copy number (CN) in KRAS wild-type xenopatients and in the quadruple negative subpopulation. EGFR copy number was arbitrarily defined as a gain when more than three EGFR
gene copies were detected by qPCR analysis on genomic DNA. Differences in distribution were statistically significant both in KRAS wild-type xenopatients \( (p < 0.001 \) by Chi-square test) and in the quadruple negative subpopulation \( (p < 0.05 \) by Chi-square test). C, Expression analysis of amphiregulin (AREG) and epiregulin (EREG) in 54 KRAS wild-type tumors. When combining tumors that responded to cetuximab with disease regression or stabilization, the median expression of AREG was significantly higher than in the group of tumors that progressed. EREG was also more expressed in tumors that responded to cetuximab with shrinkage or stabilization than in resistant cancers, but this difference did not reach statistical significance. ** \( p < 0.01 \) by two-tailed Student’s \( t \) test. R, regression; S, stabilization; P, progression.

**Figure 4. Correlation between HER2 amplification and therapeutic resistance to cetuximab in xenopatients**

A, Distribution of HER2 expression levels in a series of 137 mCRC samples, as assessed by oligonucleotide microarrays. HER2 expression in the outliers was more than two standard deviations from the mean. B, HER2 expression (HercepTest, upper images) and HER2 gene copy number (FISH, lower images) in representative cases extracted from tissue microarray analysis of an independent series of 112 mCRC archival specimens. C, Evidence of HER2 amplification in our series of cetuximab-resistant, quadruple negative tumors. Left panel: qPCR gene copy number analysis. Dotted line indicates two copies. Right panel: FISH analysis of HER2 amplification in a cetuximab-resistant, quadruple negative case (M077). Shown are samples from the original liver metastasis and from the mouse xenograft. The HER2 gene is indicated by red dots, while the control chromosome 17 centromeric probe (CEP17) is labeled in green D, Genotype-response correlations in the KRAS wild-type subpopulation as previously shown in Fig. 3A. Light grey histograms indicate cases with rare mutations of KRAS or mutations of NRAS, BRAF and PIK3CA.

**Figure 5. Correlation between HER2 amplification and therapeutic resistance to cetuximab in human patients.**

A, HER2 expression (HercepTest, upper images) and HER2 gene copy number (FISH, lower images) in representative cases of cetuximab-resistant, KRAS wild-type tumors in human patients. Patients evaluated in this cohort were selected based on evidence that treatment outcome could be attributable only to administration of either cetuximab or panitumumab. For those patients who progressed on irinotecan-based chemotherapy, cetuximab was administered in combination with irinotecan given at the same dose and schedule previously used. Clinical response was assessed with radiological examination (computerized tomodensitometry or magnetic resonance imaging). The Response Evaluation Criteria in Solid Tumors (RECIST 1.1) were adopted for evaluation. B, Prevalence of HER2 amplification in unselected mCRC patients, according to published information
as well as data from our tissue microarray analysis (42-44) (left), and in cetuximab-resistant, genetically selected patients (middle) and xenopatients (right). \( p \) values were calculated by the two-tailed binomial distribution test.

**Figure 6. Effect of anti-EGFR and anti-HER2 therapies in cetuximab-resistant, HER2-amplified mCRC xenopatients**

A, B, Growth curves of tumors in xenopatients derived from cetuximab-resistant, quadruple negative, HER2-amplified cases M077 (A) and M091 (B) (\( n = 5 \) for each treatment arm). C, Immunohistochemistry assessment with the indicated antibodies of representative tumors from case M077 at the end of treatment. Bar, 100 \( \mu \)m.
Table 1. Summary of the clinical and molecular characteristics for the study cohort

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Bertotti et al., Figure 1
Bertotti et al., Figure 3
Figure 4

A) HER2 expression (Log2 median centered) with outliers indicated.

B) Comparison of IHC-HER2 and FISH-HER2.

C) HER2 copy number distribution.

D) Variation from baseline (% of volume) with quadruple negative and HER2 amplification.
Bertotti et al., Figure 5

A

IHC - HER2

FISH - HER2

B

Unselected (n=2349) (patients)

KRAS wild type (n=44) (patients)

Quadruple negative (n=11) (xenopatients)

HER2 amplification
A molecularly annotated platform of patient-derived xenografts ('xenopatients') identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer

Andrea Bertotti, Giorgia Migliardi, Francesco Galimi, et al.

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