A Personalized Preclinical Model to Evaluate the Metastatic Potential of Patient-Derived Colon Cancer Initiating Cells

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Abstract

**Purpose:** Within the aim of advancing precision oncology, we have generated a collection of patient-derived xenografts (PDX) characterized at the molecular level, and a preclinical model of colon cancer metastasis to evaluate drug-response and tumor progression.

**Experimental Design:** We derived cells from 32 primary colorectal carcinomas and eight liver metastases and generated PDX annotated for their clinical data, gene expression, mutational, and histopathological traits. Six models were injected orthotopically into the cecum wall of NOD-SCID mice in order to evaluate metastasis. Three of them were treated with chemotherapy (oxaliplatin) and three with API2 to target AKT activity. Tumor growth and metastasis progression were analyzed by positron emission tomography (PET).

**Results:** Patient-derived cells generated tumor xenografts that recapitulated the same histopathological and genetic features as the original patients' carcinomas. We show an 87.5% tumor take rate that is one of the highest described for implanted cells derived from colorectal cancer patients. Cecal injection generated primary carcinomas and distant metastases. Oxaliplatin treatment prevented metastasis and API2 reduced tumor growth as evaluated by PET.

**Conclusions:** Our improved protocol for cancer cell engraftment has allowed us to build a rapidly expanding collection of colorectal PDX, annotated for their clinical data, gene expression, mutational, and histopathological statuses. We have also established a mouse model for metastatic colon cancer with patient-derived cells in order to monitor tumor growth, metastasis evolution, and response to treatment by PET. Our PDX models could become the best preclinical approach through which to validate new biomarkers or investigate the metastatic potential and drug-response of individual patients. *Clin Cancer Res;* 19(24): 6787–801. ©2013 AACR.

Introduction

Colorectal cancer is the second leading cause of death from cancer worldwide (1). Although surgical resection combined with adjuvant therapy is mostly effective at the early stages of the disease, both subsequent relapse and diagnosis at late stage with metastasis are frequent and responsible for the majority of patient deaths. At these advanced stages, resistance to conventional therapies are frequent and treatments are therefore quite ineffective (2). A new generation of target-directed drugs has being designed to overcome such resistance. However, a better understanding of the mechanisms driving drug-response and metastasis is crucial in order to better guide treatment decisions and improve patient outcomes.

Although the combination of 5-fluorouracil (5-FU) with oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) constitutes the basis of current treatments for metastatic colorectal cancer, the use of biologics directed to block some altered oncogenic pathways has also proven beneficial for patients with advanced colorectal cancer. Cetuximab is an antibody that specifically blocks epidermal growth factor receptor (EGFR) oncogenic signaling in cancer cells. It significantly improves the response of patients with advanced colon cancer to conventional chemotherapy increasing their overall survival (3, 4). Patients that present K-RAS–activating
mutations are refractory to Cetuximab, indicating the relevance of genotyping tumors in order to select the most appropriate personalized treatment (5, 6).

It is clear that a stepwise accumulation of particular genetic alterations is a driving force in tumor progression and may also define the resistance or sensitivity to specific target-directed drugs. The establishment of improved preclinical models that recapitulate the human disease preserving its intratumoral cell heterogeneity and histopathological and genetic alterations has become essential for testing the efficacy of new target-directed therapies and validating biomarkers of drug response. Few collections of subcutaneous patient-derived xenografts (PDX) from colorectal cancer patients have been described to date (7, 8). They recapitulate the major histological features and genetic alterations of the original patient’s carcinoma and reproduce the KRAS-dependent response to anti-EGFR drugs.

Most in vivo models developed thus far with patient-derived cells involve their transplantation to immunodeficient mice either by subcutaneous injection or into the kidney capsule (9, 10). The accessibility of subcutaneous tumors constitutes a great advantage in both monitoring tumor growth and assessing the effects of therapeutic intervention. However, a major disadvantage is that the subcutaneous microenvironment differs greatly from that of the colon. Interactions between the host environment and the tumor graft determine tumor cell expression profiles, levels of growth factors and nutrients, as well as tumor angiogenesis and metastatic behavior (11). Consequently, these models do not recapitulate the advanced stages of colon cancer because mice do not develop metastasis. The closest models to human disease involve the injection of colon cancer cell lines into the cecum wall or the transplantation of pieces of tumoral tissue derived from patients in the colon serosa of nude mice. Concerning the latter, cells can metastasize to the lymph nodes, liver, and peritoneum, but they do not generate lung metastasis (12). Finally, freezing tissue pieces reduces cell viability, making the generation of a patient-derived cells collection for long-term studies difficult.

Orthotopic injection of cancer cell lines can also recapitulate the metastatic dissemination to the main tissues affected in patients with advanced colorectal including lung metastasis (13, 14). However, although cell lines are able to self-renew, they lose their pluripotency, generating very homogeneous tumors which do not recapitulate the cell heterogeneity characteristic of human colon carcinomas (15). This is an important factor that may partly explain the lack of correlation between the in vivo response of cell lines to antitumoral drugs and the resistance of patients to equivalent treatments. The discrepancies are even more important when cell lines are injected subcutaneously, observing in many cases a positive response to antitumoral agents in preclinical models that clearly fail in clinical trials where patients’ disease progresses (15).

Several positron emission tomography (PET) studies have been reported in mouse models of human cancer (16, 17). The most frequently employed radiotracer was 18F-fluorodeoxyglucose (18F-FDG) followed by 18F-fluorothymidine (18F-FLT, ref. 18). Both have been used to identify the tumoral lesion, follow their growth and evaluate the effectiveness of new treatments in vivo (19, 20). To our knowledge, there have been no other previously reported PET studies in mice inoculated orthotopically with patient-derived colon cancer cells. We also aimed to establish which is the most advantageous radiotracer to evaluate colon cancer in mice.

In summary, currently available models of colon cancer have produced incomplete results, misguiding oncologists and pharmaceutical companies when important decisions are taken based on such preclinical data. This misleading information can affect the initiation, design, or evolution of clinical trials with new antitumoral drugs and consequently the future of patients affected by advanced colon cancer. This unacceptable scenario calls for an accurate preclinical model that faithfully recapitulates metastatic colon cancer in order to evaluate the potential benefit of new drugs for patients with advanced disease more precisely.

We have generated a PDX collection from primary colon carcinomas and liver metastasis annotated for gene expression, mutational status, histopathological, and clinical data. Our rapidly expanding collection is incorporating the main colorectal tumor subtypes allowing us to test the efficacy of target-directed drugs. We have also developed a preclinical model of colon cancer metastasis by injecting patient-derived colon cancer cells into the cecum wall of NOD-SCID mice and following tumor evolution by PET. Functional evaluation of treatment response could be performed with cells derived from individual patients, providing precise experimental data to oncologists upon selecting the best tailored therapy.
Materials and Methods

Tissue collection and patient information
Written informed consent was signed by all patients. The project was approved by the Research Ethics Committee of the Vall d’Hebron University Hospital, Barcelona, Spain (approval ID: PR(IR)79/2009). Human colon tissue samples consisted of biopsies from nonneoplastic areas of primary adenocarcinomas or liver metastases corresponding to patients with colon and rectal cancer who underwent tumor resection. Sections for immunohistochemistry were obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks. Detailed clinico-pathological information including tumor location, TNM (tumor, nodes, metastasis) status was compiled and genetic analysis carried out for each patient. Histological diagnosis, evaluated by the Pathology Service of Vall d’Hebron University Hospital, was based on microscopic features of carcinoma samples determining the tumor histotype, grade, and stage. The assessment of nuclear expression of mismatch-repair proteins (MLH1, MSH2, MSH6, PMS2) was performed by immunohistochemistry.

Gene expression
Hematoxylin and eosin (H&E) staining was performed in each FFPE tumor tissue. Areas enriched in tumor tissue were identified. A minimum of two 1-mm FFPE tumor tissue cores were collected. RNA was purified using the Roche HighPure FFPE Micro Kit, and ~100 ng of total RNA was used to measure expression of 292 selected genes using the nCounter platform from Nanostring Technologies (www.nanostring.com). In short, fluorescently labeled probes are designed for specific genes and allowed to hybridize to target RNAs, and then captured and individual RNA molecules counted using color-coded probe pairs (21). Raw data were log base 2 transformed and normalized using 5 housekeeping transcripts.

Mutational status
Human tumor samples were genotyped as previously described (22). Microsatellite instability was analyzed using the MSI-Analysis System (Promega).

Patient-derived cells isolation and culture
Colon carcinoma tissues obtained upon surgery were washed 3 times in cold PBS solution and incubated overnight in DMEM/F12 (Gibco) containing a cocktail of antibiotics (100 U/mL; Sigma–Aldrich) and antifungals (as described above) during 1 hour at 37°C with intermittent pipetting every 15 minutes to disperse cells. The dissociated sample was then filtered (100 μm pore size) and washed with fresh medium. Red blood cells were lysed by brief exposure to ammonium chloride and the sample was washed again. Finally, cells were used for subcutaneous or orthotopic injections in NOD-SCID mice.

Xenograft tumors in mice
Experiments were conducted following the European Union’s animal care directive (86/609/EEC) and were approved by the Ethical Committee of Animal Experimentation of the VHIR–the Vall d’Hebron Research Institute (ID: 40/08 CEEA and 47/08/10 CEEA). NOD-SCID (NOD. CB17-Prkdcscid/NcrCrl) were purchased from Charles River Laboratories. A total of 1 × 10⁵ patient-derived cells suspended in PBS were mixed 1:1 with Matrigel (BD Bioscience) and injected subcutaneously into both flanks of NOD-SCID mice. After 3 to 8 weeks, visible tumors were detected. When the tumor grew to 1 cm³ in size, mice were euthanized and xenografts were processed to obtain a new cell suspension as previously described or fixed for histological analysis.

For orthotopic transplantation, 1 × 10⁶ patient-derived cells suspended in 50 μL of PBS were injected into the cecum wall of NOD-SCID mice as has been previously reported (14). API-2 (Tocris Bioscience) at 1 mg/kg in PBS + 2% dimethyl sulfoxide (DMSO) or oxaliplatin (Sigma) at 20 mg/kg in PBS were injected intraperitoneally every second day or twice a week respectively beginning the first day of tumor detection by palpation. Control mice were injected with the corresponding amount of vehicle (PBS + 2% DMSO or PBS). In the case of API-2–treated animals, tumor growth was measured by micro-PET imaging. Tumor growth was assessed by palpation in oxaliplatin-treated animals. When matching endpoint criteria mice were euthanized and complete necropsies were performed. Primary carcinomas in the cecum and metastases in the liver, lung, or any other visible tissue affected were collected for histological analysis.

Immunohistochemistry and antibodies
All immunostaining was performed on paraffin-embedded tissues. Tissue blocks were sectioned, mounted on microscope slides, and heated at 56°C overnight. Paraffin was removed with xylene and tissues were serially rehydrated through descending ethanol concentrations to water. Sections were stained with H&E to assess cellular morphology. For immunofluorescence, antigen retrieval was performed by boiling the samples in a microwave oven using 10 mmol/L sodium citrate buffer (pH 6). Slides were then washed twice in PBS and once in PBS-1% Tween-20 (Sigma-Aldrich) for 15 minutes. Tissue specimens were blocked for 1 hour with PBS containing 3% of bovine serum albumin. Slides were incubated with specific primary antibodies at 4°C overnight: β-catenin 1:100 (Abcam), caspase-3 1:100 (Cell Signaling Technology), chromogranin A 1:100 (Clone LK2H10; AbDSerotec), cytokeratin 20 1:100 (Clone Ks, 20.8; Dako), EpCAM 1:100 (Clone E144; Abcam), Ki67 1:100 (DAKO 1:100).
Cytomation), MJIC2 1:100 (Clone MOPC-21; BD Bioscience), and Villin1 1:100 (Lifespan Bioscience). Double immunostaining was performed incubating slides with the corresponding secondary antibodies (goat anti-mouse and goat anti-rabbit) conjugated to Alexa Fluor 488 and Alexa Fluor 555 (Invitrogen) at a dilution of 1:200 for 1 hour at room temperature. Nuclei were stained with Hoechst 33342 (5 μg/mL; Sigma-Aldrich). An Olympus FluoView FV1000 Confocal Microscope was used to visualize fluorescence and acquire images.

**Positron emission tomography**

Mice were anesthetized in an induction cage using isofluorane vaporized in O2 at a concentration of 4%. After the anesthesia induction, animals were placed in a lateral position and received the radiotracer dose in one of the tail veins. After dose injection, the animals were returned to their cages for 2 hours’ radiotracer uptake. In the case of 11C-methionine and 11C-choline, the image started immediately after radiotracer injection. During PET acquisition, mice were kept under anesthesia with isofluorane at 1% in O2 vaporized through orofacial masks. Injected doses were (mean ± SD) 271 ± 88.8 mCi for the 18F-FDG, 186.1 ± 40.5 mCi for the 18F-FLT, 294.2 ± 106.9 mCi for the 11C-choline, and 216.8 ± 91.4 mCi for 11C-methionine.

Emission data were acquired for 30 minutes in a microPET R4 system (Concorde 175 Microsystems; Siemens). Data were corrected for nonuniformity, random coincidences, and radionuclide decay, but not for scatter or attenuation. 11C studies were reconstructed with a filtered back-projection algorithm into a matrix size of 128 × 128 × 63, a voxel size of 0.85 × 0.85 mm, and slice thickness of 1.21 mm. 18F studies were reconstructed with a OSEM-2D algorithm into a matrix size of 256 × 256 × 63 and a voxel size of 0.42 × 0.42 × 1.21.

Images were visually inspected and when a tumor was clearly identified, volumes of interest were then defined. Finally, in the images obtained with 18F-FDG, the volume of tumoral tissue was calculated in order to follow the grown of the mass over time.

**Statistics**

Differences in primary tumor or metastases parameter classes were analyzed by Fisher and χ² tests. Differences in proliferation and apoptosis status were analyzed by unpaired t test with Welch correction. Differences in tumor volume were analyzed by unpaired t test comparing the means of untreated and API2-treated groups of values. In all cases, a P value lower than 0.05 was considered statistically significant.

**Results**

**Engraftment of colorectal patient-derived cells**

All patient-derived tumor tissues were disaggregated in a single-cell suspension and a minimum of 1 × 10⁷ viable cells were subcutaneously injected into both flanks of NOD-SCID mice. Consecutive cell purification and reinjection was performed from the initial PDX tumors to amplify the samples in a second generation of mice. The 40 PDX shown (Tables 1 and 2) are part of an expanding collection of models stored as frozen single-cell suspensions, which can be thawed and reinjected in NOD-SCID mice without any reduction of their tumor take rate.

A total of 32 primary colorectal cancer carcinomas (Table 1) and 8 liver metastases (Table 2) were processed and implanted in NOD-SCID mice. A total of 27 primary carcinomas and all 8 metastases engrafted and generated a PDX model, representing an overall 87.5% tumor take rate (Tables 1 and 2). This is one of the highest tumor take rates ever described for colorectal cancer patient-derived xenografting (7–9). A total of 4 of the 5 failed engraftments corresponded to cells derived from patients with no lymph nodes affected (N0) presenting nonmucinous adenocarcinomas. Furthermore, the tumor take rate of the successfully engrafted N0 tumors was significantly lower than N+ tumors (P = 0.0117). We also observed a reduced engraftment capacity of tumors with lower differentiation grade G1. The only 2 G1 tumors processed failed to grow in mice (P = 0.0206). No other statistically significant correlation was observed with the rest of the patients’ clinicopathological characteristics.

Cells derived from all liver metastasis samples generated PDX, indicating their enhanced engraftment potential as previously suggested (7, 25, 26). Liver-derived tumor cells from advanced patients treated with adjuvant chemotherapy showed a lower implantation rate, although not a statistically significant difference (Table 2). Furthermore, metastasis-derived PDX showed a shorter xenograft latency time (46.6 ± 21.7 days) than those derived from primary carcinomas (68 ± 34 days).

**Histological and molecular characterization of the PDX collection**

The histopathology of all PDX (first and second passage in mice) presented in concordance with their respective patient’s original carcinoma (Supplementary Fig. S1A and S2B and data not shown). The collection included 8 models derived from mucinous adenocarcinomas and 27 conventional adenocarcinomas. Samples P10 (primary mucinous adenocarcinoma) and P33 (liver metastasis) were derived from the same patient who underwent surgery upon initial diagnosis of colorectal cancer and then after relapse with liver metastasis. This patient is the only rectal carcinoma case in our PDX collection. Because gene expression profiles can define a particular tumor subtype (27–29), we analyzed 33 of the 35 established PDX using the Nanostring platform with an identifier of 292 genes (Fig. 1 and Supplementary Table S1). Sample preparation failed in the 2 remaining PDX. We observed a perfect clustering of all mucinous separated from the nonmucinous adenocarcinomas. P10 and P33 samples derived from the same patient clustered together indicating their similarities at gene expression level.
All 35 PDX samples were also genotyped using Sequenom technology to identify the most frequent mutations in oncogenes and tumor suppressors (ref. 22; Fig. 1 and Supplementary Table S2). We detected mutations frequent in colorectal tumors affecting KRAS, PIK3CA, APC, TP53, or BRAF (29). E542K or E455K mutations in PIK3CA gene were frequent in PDX derived from conventional carcinomas and absent from mucinous

Table 1. Patients’ clinical characteristics and PDX implantation rates of primary tumors

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<th>Number (%)</th>
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<td>pM1</td>
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Cells derived from primary colorectal tumors presented different implantation rate or tumor latency when injected subcutaneously in NOD-SCID mice. Histological differentiation grade (G1–G2, well/moderate; G3–G4, poor/undifferentiated). Tumor-node-metastasis status. pT1–pT4, invasive tumors (pT1, submucosa; pT2, tunica muscularis; pT3, subserosa; pT4, serosa or other organs). pN0, no malignant lymph nodes; pN+, at least 1 positive regional lymph node. pM0, no distant metastasis; pM1, presence of distant metastasis; Unknown, at the stage of primary tumor evaluation, metastasis cannot be assessed. Cases included in this study were reported as having been staged according to 7th edition of the American Joint Committee on Cancer (AJCC) Staging Manual (2010). Asterisk indicates a significant difference (P < 0.05) between groups as quantified by Fisher test or $x^2$ test.

All 35 PDX samples were also genotyped using Sequenom technology to identify the most frequent mutations in oncogenes and tumor suppressors (ref. 22; Fig. 1 and Supplementary Table S2). We detected mutations frequent in colorectal tumors affecting KRAS, PIK3CA, APC, TP53, or BRAF (29). E542K or E455K mutations in PIK3CA gene were frequent in PDX derived from conventional carcinomas and absent from mucinous
adenocarcinomas. Mutation in PIK3CA at position H1047R was only observed in samples 10 and 33 from the patient with rectal cancer. No other correlation was observed between mutational status, gene expression clusters, and histopathological characteristics. Paired genotyping was performed on 12

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Cells derived from liver metastasis presented different implantation rate or tumor latency when injected subcutaneously in NOD-SCID mice. Histological differentiation grade (G1–G2, well/moderate; G3–G4, poor/undifferentiated). Tumor-node-metastasis status. pT1–pT4, invasive tumors (pT1, submucosa; pT2, tunica muscularis; pT3, subserosa; pT4, serosa or other organs). pN0, no malignant lymph nodes; pN+, at least 1 positive regional lymph node; pM0, no distant metastasis; pM1, presence of distant metastasis; Unknown, at the stage of primary tumor evaluation, metastasis cannot be assessed. Cases included in this study were reported as having been staged according to 7th edition of the American Joint Committee on Cancer (AJCC) Staging Manual (2010). Asterisk indicates a significant difference (P < 0.05) between groups as quantified by Fisher test or χ² test.
original tumor samples obtained upon surgery and those from the corresponding PDX. In all cases, we observed a perfect match between the mutations detected in the original tumors and their paired PDX.

**Metastatic potential of colorectal cancer patient-derived cells**

We derived tumor cells from colon carcinomas surgically removed from 6 different patients (Table 3). Patient 1 (sample P1) presented a pT4aN0 high-grade exophytic mucinous adenocarcinoma that invaded adjacent adipose tissue (reaching the serosa), but none of the 20 lymph nodes analyzed. Patient 1 received 6 months' Capecitabine as adjuvant therapy following surgery. Patient 2 (sample P2) developed a pT4aN1 high-grade conventional adenocarcinoma that infiltrated the intestinal serosa and 2 of 29 lymph nodes. Patient 3 (sample P3) presented a pT3N1a low-grade conventional adenocarcinoma that infiltrated serosa, adipose tissue, and 1 of 15 lymph nodes. Patient 5 (sample P5) presented a high-grade, poorly differentiated, pT4aN0 mucinous carcinoma that invaded the adipose tissue but none of the 16 lymph nodes analyzed. Patient 5 received De Gramont adjuvant chemotherapy for 6 months after surgery. Patient 6 (sample P6) presented a high-grade, pT4aN2b, ulceroinfiltrative mucinous adenocarcinoma that invaded the serosa and 7 of the 27 lymph nodes analyzed. This patient relapsed 5 months after initial surgery presenting infiltration in lymph nodes and metastases in the liver and the adrenal gland. Patient 10 of 33 presented a low-grade, pT4aN0, primary mucinous adenocarcinoma in the rectum that invaded the serosa but none of the 14 lymph nodes analyzed (sample P10). Patient 10 of 33 received FOLFOX chemotherapy for 5 months but relapsed 16 months after the initial surgery, developing polylobulated liver metastasis (sample P33). Patient 2 died because of surgical complications and patient 5 died 5 months after diagnosis of relapse. The other 4 patients are still alive and have not, up until now, developed distant metastases, as observed by thoraco-abdominal computerized tomography.

A total of $1 \times 10^6$ cells derived from each patient were injected into the cecum wall of 10 NOD-SCID mice to evaluate their metastatic potential. A few mice died unexpectedly a couple of days after injection because of surgical complications. All mice injected with cells derived from patient 1 generated a primary adenocarcinoma in the cecum that recapitulated the same histology as the original patient’s tumor and subcutaneous xenograft, demonstrating high cancer initiation potential and pluripotency capacity (Table 4 and Supplementary Fig. S1A). Equivalent results were obtained with cells derived from patients 2, 6, and 33 with a 100% tumor take rate, whereas those from patient 3 generated tumors in 3 of the 5 injected mice. Regarding the cells from patient 3, injection into the cecum wall was performed just after thawing the cells, validating the fact that the freezing process still preserved most of their tumorigenic potential. Finally, 8 of 9 mice

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**Figure 1.** Hierarchical cluster based on gene expression of primary tumors and metastases. Cells derived from each PDX were analyzed for gene expression of 293 genes using the nanostring platform. Tumor samples were ordered by hierarchical clustering using uncentered Pearson correlation distance and complete linkage with Cluster 3.0 software (43). The cluster tree was visualized with GenePattern tools (http://genepattern.broadinstitute.org) and represents the relative distance similarities of samples. The same samples were also genotyped by Sequenom using a panel of frequent mutations in oncogenes and tumor suppressor genes (22).
generate a tumor in the cecum when injected with cells derived from patient 5.

Cells derived from the mucinous adenocarcinomas of patients 1 and 5 generated metastases in the abdominal cavity (carcinomatosis), lungs, and liver that were confirmed by H&E staining and immunofluorescence for EpCAM and CK20 (Table 4, Fig. 2 and Supplementary Fig. S2). On the contrary, cells derived from patients 2 and 33 did not generate metastases in any of the injected mice. In the case of patient 3, 1 mice out of 3 injected developed carcinomatosis and lung metastasis. In the case of cells derived from patient 6, injected mice died unexpectedly and distant tissues could not be collected in order to determine the presence of metastases.

The pluripotency of patient-derived cells was evaluated in further detail by staining patients’ original adenocarcinomas and the corresponding xenograft tumors for lineage differentiation markers. The 6 PDX models recapitulated the same differentiation heterogeneity as the original carcinoma presenting particular proportions of mucinosecretory (Mucin 2), absorptive (Vilin 1), or enteroendocrine (Chromogranin) cells (Supplementary Fig. S3A and S3B).

Testing drug response in colorectal cancer PDX models

We first studied the effect of oxaliplatin chemotherapy on tumor growth and metastasis using our orthotopic PDX model. We injected cells derived from patients 5, 6, or 33 into the cecum wall of 10 NOD-SCID mice. A few mice died unexpectedly a couple of days after injection because of surgical complications. Two months after injection, half the mice were treated every second day with oxaliplatin and the other half with vehicle until sacrificed. Only mice injected with cells from patient 5 and treated with vehicle generated distant metastases in the lungs and liver (3 of 4 mice), whereas oxaliplatin prevented metastases formation (4 of 4 mice). Mice injected with cells from patient 33 did not generate distant metastasis irrespective of treatment. Finally, mice injected with cells from patient 6 died unexpectedly and tissues could not be collected for histological evaluation of distant metastasis.

To further evaluate the effect of treatment with oxaliplatin, we quantified the presence of proliferative (Ki67) or apoptotic (cleaved caspase 3) cells on histological sections of primary xenograft tumors growing in the cecum of NOD-SCID mice (Fig. 3A). We observed a significant effect of oxaliplatin on the proliferation of PDX from patient 33 and a trend in those derived from patient 5. In addition, apoptosis was increased in tumor xenografts from patient 5 and no effect was observed in those from patient 33.

We also used our colorectal cancer metastasis model and PET to evaluate the response to API2, a target-directed drug that inhibits AKT activity. We first evaluated different radiotracers in our mouse model to observe tumor growth and metastasis by PET (Supplementary Fig. S4). 18F-FLT presented a very high uptake by normal intestinal mucosa, especially in the colon. In addition, 18F-FLT is highly excreted into the urine producing a high radioactivity.
patient 2, was observed upon API-2 treatment (Fig. 3D). A 2 cohort compared with vehicle-treated mice. On the con-
ting from the primary tumor growing in the cecum wall
carcinomatosis were detected by PET when located dis-
nificantly from the primary tumor growing in the cecum wall
of biomarkers of response. It is becoming evident that the identification of robust biomarkers to
predict response to treatment is essential for the success of clinical trials with target-directed drugs. Although some of
these novel drugs are showing promising results in advanced cancer, resistance is frequent in most metastatic
colorectal patients. There is therefore an urgent need for preclinical models that permit the testing of the efficacy of this new generation of target-directed drugs and validation of biomarkers of response.

To overcome resistance to conventional treatments, numerous drugs blocking specific molecular targets have been developed over the last decade and are currently being tested on patients with different tumor types. It is becoming evident that the identification of robust biomarkers to predict response to treatment is essential for the success of clinical trials with target-directed drugs. Although some of these novel drugs are showing promising results in advanced cancer, resistance is frequent in most metastatic colorectal patients. There is therefore an urgent need for preclinical models that permit the testing of the efficacy of this new generation of target-directed drugs and validation of biomarkers of response.

Historically, most preclinical studies have been based on cell line models in vitro and in vivo. However, the long-term growth of cell lines selects a homogenous population that is the most efficient in proliferating in a particular culture condition. One of the most important defects of such models is that the response to antitumoral drugs is not representative of what actually occurs in heterogeneous human carcinomas (15). It is well accepted that intratumor heterogeneity occurs with respect to a variety of biological, biochemical, and immunological properties (30). These properties determine the ability of particular cancer cell subpopulations to emerge from the primary tumor and establish metastatic growth within distant organs (31). Furthermore, resistance to specific treatments can also be innate in some genetic subclones present in polyclonal colorectal carcinomas (23).

The preservation of patients’ intratumoral heterogeneity at the cellular and genetic levels is majorly improved in PDX...
models compared with preclinical mouse models based on cancer cell lines. Therefore, PDX are currently becoming the best preclinical models to test drug response.

We have established a circuit to derive cancer cells just after surgical removal of colorectal tumors. We first disaggregate the patient’s tumor piece, prepare a suspension of single cells, and then subcutaneously inject a minimum of $1 \times 10^5$ viable cells in NOD-SCID mice, all within less than 24 hours. This procedure has resulted in an 87.5% tumor take rate, which is higher than the average 60% previously described in colorectal cancer PDX collections (7–9). Most of the laboratories that have generated similar
Figure 3. Response of orthotopic colorectal cancer PDX to chemotherapy and API2 treatment. A (top), cecum primary xenograft tumors developed from P5 and P33 treated with oxaliplatin or vehicle were immunostained for Ki67 (red) and Caspase-3 (green). Representative confocal pictures are shown. Scale bar, 100 μm; (bottom) column scatter plot showing the amount of Ki67 (proliferation) or caspase-3 (apoptosis) in cecum xenografts from P5 and P33 treated with oxaliplatin (red) or vehicle (green). Horizontal lines indicate arithmetic mean values, and error bars show the 95% CI. Asterisk indicates a significant difference ($P < 0.05$) between groups as quantified by unpaired t test with Welch correction. r.u., relative units. B, representative 18FDG PET images of mice injected with cells derived from P1, obtained at baseline and after 10, 30, and 60 days of vehicle or API-2 treatment. H, heart; B, urinary bladder. Dashed lines delineate primary xenograft tumors growing in the cecum wall. C, plot representing the evolution of tumor xenograft volume growing in mice injected with cells derived from P1 and treated with API-2 or vehicle. D, plot showing the final tumor xenograft volume after 48 days of treatment with API-2 or vehicle in mice injected with cells derived from P1 (green bars) or P2 (red bars). Statistical significance was evaluated by unpaired t test with Welch correction.
collections of PDX models transplant a piece of tumor tissue in immunodeficient mice (7, 8). Intact human tumor tissue often contains large necrotic areas thus the number of viable cells implanted is unknown and engraftment could be compromised. Such inaccuracy would lead to a more variable tumor initiation efficacy, latency time, and growth rate, making it more difficult to compare between different experiments or even individual mice, as well as complicating the setup of robust experiments to test the activity of antitumoral agents (11). Furthermore, freezing pieces of tumor tissue compromises cell viability more than protocols with single cells. In our models, we can freeze and therefore perpetuate each patient sample for future tests when required, better preserving their capacity to reinitiate an equivalent xenograft tumor.

In addition, different tumor pieces from the same patient could be enriched for particular genetic or epigenetic subclones and their separated implantation would generate tumor xenografts with different biological properties. To overcome this potential bias, we inject a suspension of disaggregated single cells ensuring a better representation of the original patients’ intratumoral heterogeneity.

Using our cell suspension protocol we observed a reduced tumor take rate from patients that presented no invaded lymph nodes (N0) or those with lower differentiation grade (G1). Similar correlations have been reported in other colorectal cancer PDX collections, indicating that less aggressive tumors have a reduced capacity to engraft in immunodeficient mice (7, 25).

The use of single cells disaggregated from patient tumor tissue, opens up many experimental avenues such as sphere culturing to test drug-response in vitro, or to purify (FACS) and study the biological characteristics of different cell subpopulations present in heterogeneous colorectal carcinomas. We have also observed that cancer initiation potential is preserved in sphere cultures of tumor cells derived for most of the patients with colorectal cancer. The PDX generated from cultured cells also preserve pluripotency because they recapitulate the same cell heterogeneity and histopathological traits as the original patients’ colorectal carcinoma (data not shown).

At the genetic level, our protocol permits the generation of tumor xenografts that present the same mutational status as the original patients’ carcinoma. It would therefore allow the study of the correlation between drug-response observed in our mouse models with the mutations present in each particular patient. Twelve original patient carcinomas and PDX pairs were genotyped presenting the same mutational pattern. Only in 2 cases was the allele frequency increased for a particular oncogenic mutation in the PDX versus the original patient’s sample (data not shown).

We could detect the most frequent mutations in genes classically altered in colorectal cancer such us KRAS, PIK3CA, BRAF, or APC. The analysis of APC is clearly incomplete because the Sequenom platform only permitted testing some of the most frequent single nucleotide mutations described. Sequencing all APC exons should be performed to identify any possible mutations affecting this tumor suppressor gene, which is essential for colorectal cancer carcinogenesis.

The genetic data generated demonstrates that our PDX models faithfully represent the main genetic characteristics of patients with colorectal cancer (7). The percentage of cases mutated for each particular gene (e.g., KRAS) in our collection differs from those previously described in larger collections of patients with colorectal cancer (29). Such discrepancy could be because of the fact that our collection is enriched in advanced tumors and liver metastasis.

Using the Nanostring platform with a discrete panel of genes, PDX samples were also evaluated for gene expression. The gene expression study allowed the clustering of PDX with similar profiles. All PDX derived from mucinous colon carcinomas clustered together, similarly to the results previously shown in larger collections of colorectal cancer samples (29). We could even observe that samples P10 and P33, corresponding respectively to the primary rectal tumor and the liver metastasis of the same patient, clustered together and separately from the rest of the PDX analyzed. Similar studies with microarrays have demonstrated that gene expression profiles from original patients’ carcinomas and different PDX passages in mice cluster. Such data evidences that the control of gene expression patterns is mostly a tumor cell autonomous trait. Such reproducibility and cell autonomous behavior in PDX models would facilitate the future use of gene expression profiles as powerful biomarkers to predict drug response or tumor progression.

In all cases, PDX models recapitulate the same histology as the original patients’ carcinoma. Therefore, tumor architecture seems to be a cell autonomous trait mainly independent of the accompanying stroma. Such characteristic of cancer cells has also been reported in other PDX collections of colorectal cancer tumors whereas patients’ stroma is replaced by an equivalent mouse stroma. Such capacity of tumor cells to educate the host stroma reinforces the strength of PDX as cancer models that faithfully recapitulate human disease. Thus, PDX models are the optimal preclinical approach to test target-directed therapies that could also affect the stromal component.

Because metastatic colorectal cancer is currently lethal for the vast majority of patients, most therapeutic efforts are now focused on testing new target-directed drugs to improve their survival. It has therefore become crucial to study the antitumoral properties of such novel drugs in models that reproduce advanced human disease. Aiming to address such an urgent need, we have developed a mouse model of colorectal cancer metastasis with patient-derived cells that recapitulates human advanced disease with great precision. It could therefore become the gold standard preclinical model to test new target-directed drugs or to validate potential biomarkers of tumor progression or response to treatment. We inject a suspension of viable single cells derived from patients with colorectal cancer into the cecum wall of immunodeficient NOD-SCID mice. Primary tumors that grow in the cecum present the same histopathological features as the original patients’
We observed that oxaliplatin treatment was able to prevent metastasis in our mouse models injected with cells derived from patient 5. Proliferation was reduced and apoptosis increased in the primary cecal tumors of mice treated with oxaliplatin chemotherapy. Curiously, although patient 5 was not treated with oxaliplatin she followed a De Gramont adjuvant chemotherapy (5-fluorouracil + folic acid) and has not relapsed with metastasis during the last 25 months after surgery (Table 3). Our results are similar to previous reports with subcutaneous PDX responding to chemotherapy (7), but our model also permits to evaluate the antimetastatic effect of treatment contrarily to subcutaneous PDX which do not produce metastases.

To test the validity of our model for investigating the activity of target-directed drugs, we treated cecum-injected mice with API2, an AKT inhibitor with proven antitumoral activity (38, 39). API2 reduced tumor growth of mucinous (patient 1) but not conventional (patient 2) adenocarcinomas. We recently described nuclear β-catenin as responsible for this differential response to treatment. High nuclear β-catenin content in patient 2 would confer resistance to FOXO3a-induced apoptosis promoted by PI3K or Akt inhibitors, whereas mucinous carcinomas from patient 1 that accumulate low nuclear β-catenin amounts are sensitive (22). The evaluation of tumor response to API2 treatment was based in PET measurements.

Conventional noninvasive imaging methods, including PET, that are routinely used in the clinic, have been adjusted to monitor tumor initiation, progression, and response to therapy in mouse models of cancer (40, 41). However, we are the first to use PET to monitor tumor xenografts from colon cancer patient-derived cells growing in the cecum of immunodeficient mice. From our studies, we can conclude that PET with 18F-FDG permits the evaluation of primary tumor localization, growth, and development of distant metastases or quantify response to treatment in our mouse model of advanced colorectal cancer with patient-derived cells. Indeed, PET is used in the clinic to detect the presence of metastasis or the response to treatments in patients with colon cancer (42).

PDX models open a promising avenue for precision oncology because functional assays could be performed with cells derived from patients with colon cancer upon surgery. For instance, patients at early stages of disease that undergo surgery to remove a primary carcinoma and that could relapse and develop metastasis in the future, could potentially benefit from these assays to test the metastatic capacity of their cells derived from the primary carcinoma. At the same time, assays to evaluate the response of xenografted tumors derived from a particular patient to antitumoral drugs could be performed just upon initial surgery or when relapse occurs. Such assays would provide valuable information about functional resistance or sensibility to approved biologics such as anti-EGFR drugs, or experimental drugs such as PI3K, AKT, MEK, or BRAF inhibitors currently tested in early-stage clinical trials. This functional information is perfectly complementary to the particular mutation status of each patient with colon cancer, better guiding therefore oncologists to select the best targeted therapy.
Conclusions

We present an improved procedure to generate colorectal cancer PDX with a high tumor take rate that has allowed the generation of a rapidly expanding collection of models for further preclinical studies. The gene expression profiling, genotyping, and histological description of each PDX would allow the study of the strength of new biomarkers of tumor progression or response to treatment, as well as test the efficacy of new target-directed drugs.

We have also generated a mouse model of metastatic colorectal cancer with patient-derived cells that allows evaluating their metastatic potential and response to treatment. Using this approach we have generated a model that also recapitulates advanced human disease at the genetic and histopathological level.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


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