A Personalized Pre-clinical Model to Evaluate the Metastatic Potential of Patient-derived Colon Cancer Initiating Cells

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TRANSLATIONAL RELEVANCE

Metastatic colorectal cancer patients present enhanced resistance to conventional chemotherapy or target-directed drugs and a high rate of mortality. It is therefore critical to establish pre-clinical models characterized at molecular level, to test the efficacy of new therapies and functionally validate biomarkers of response. Translating experimental results from these models to the clinic will facilitate the identification of drug-sensitive patients and better guide therapy selection.

We have developed an improved procedure to generate patient-derived xenograft (PDX) models of colorectal cancer that retain the histological and genetic traits of the original patients’ carcinomas. We have also established an orthotopic model of colorectal metastasis by injecting tumor cells into the cecum wall of NOD-SCID mice. Our model allows us to monitor drug-response and metastatic capacity of colon cancer patient-derived cells and potentially correlate the results with distinctive molecular biomarkers.
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 pre-clinical 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.

Conclusion: 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 pre-clinical approach through which to validate new biomarkers or investigate the metastatic potential and drug-response of individual patients.
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 CRC. Cetuximab is an antibody that specifically blocks EGFR oncogenic signalling in cancer cells. It significantly improves the response of advanced colon cancer patients 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 pre-clinical models that recapitulate the human disease preserving its intra-tumoral 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 PDX from CRC 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 behaviour (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 advanced colorectal patients 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 anti-tumoral 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 anti-tumoral agents in
pre-clinical models which 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 $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) followed by $^{18}$F-fluorothymidine ($^{18}$F-FLT) (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 pre-clinical data. This misleading information can affect the initiation, design or evolution of clinical trials with new anti-tumoral drugs and consequently the future of patients affected by advanced colon cancer. This unacceptable scenario calls for an accurate pre-clinical 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 pre-clinical 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 non-necrotic areas of primary adenocarcinomas or liver metastases corresponding to colon and rectal cancer patients that underwent tumor resection. Sections for immunohistochemistry were obtained from formalin-fixed paraffin-embedded tissue blocks. Detailed clinico-pathological information including tumor location, TNM status (Tumor, Nodes, Metastasis) 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 formalin-fixed paraffin-embedded (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 was log base 2 transformed and normalized using five house-keeping 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 three times in cold PBS solution and incubated overnight in DMEM/F12 (Gibco) containing a cocktail of antibiotics and antifungals (penicillin (250 U/ml), streptomycin (250 µg/ml), fungizone (10 µg/ml), kanamycin (10 µg/ml), gentamycin (50 µg/ml) and nystatin (5 µg/ml) (Sigma-Aldrich)). Isolation of patient derived cells has been previously described (23-25). Enzymatic digestion was performed using collagenase (1.5 mg/ml; Sigma-Aldrich) and DNase I (20 µg/ml; Sigma-Aldrich) in a medium supplemented with a cocktail of antibiotics and antifungals (as described above) during 1 h 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-Prkdc^{scid}/NcrCrl) were purchased from Charles River Laboratories. 1x10^5 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 3-8 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, 1x10⁶ patient-derived cells suspended in 50 µl of PBS were injected into the cecum wall of NOD-SCID mice as has been previously reported (25). API-2 (Tocris Bioscience) at 1 mg/kg in PBS + 2% 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 end-point 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 re-hydrated through descending ethanol concentrations to water. Sections were stained with Haematoxylin and Eosin (H/E) to assess cellular morphology. For immunofluorescence, antigen retrieval was performed by boiling the samples in a microwave oven using 10 mM sodium citrate buffer (pH 6). Slides were then washed twice in PBS and once in PBS-1% Tween-20 (Sigma-Aldrich) for 15 min. Tissue specimens were blocked for 1 h with PBS containing 3% of BSA. Slides were incubated with specific primary antibodies at 4 °C overnight: β-catenin 1:100 (Abcam), Caspase-3 1:100 (Cell Signalling), Chromogranin A 1:100 (Clone LK2H10; AbDSerotec), Cytokeratin 20 1:100 (Clone Ks 20.8; Dako), EpCAM 1:100.
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(Clone E144; Abcam), Ki67 1:100 (DAKO Cytomation), MUC2 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 h 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.

PET

Mice were anesthetized in an induction cage using isofluorane vaporized in O2 at a concentration of 4%. After the anaesthesia 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 two 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 anaesthesia 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, Knoxville, USA). Data were corrected for non-uniformity, 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 x 128 x 63, a voxel size of 0.85 x 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 x 256 x 63 and a voxel size of 0.42 x0.42 x 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 $^{18}$F-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's and Chi-square 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 1x10^5 viable cells were subcutaneously injected into both flanks of NOD-SCID mice. Consecutive cell purification and re-injection was performed from the initial PDX tumors to amplify the samples in a second generation of mice. The 40 PDX shown (Table 1 and 2) are part of an expanding collection of models stored as frozen single-cell suspensions which can be thawed and re-implanted in NOD-SCID mice without any reduction of their tumor take rate.

32 primary CRC carcinomas (Table 1) and eight liver metastases (Table 2) were processed and implanted in NOD-SCID mice. 27 primary carcinomas and all eight metastases engrafted and generated a PDX model, representing an overall 87.5 % tumor take rate (Table 1 and 2). This is one of the highest tumor take rates ever described for CRC patient-derived xenografting (7-9). 4 out of the 5 failed engraftments corresponded to cells derived from patients with no lymph nodes affected (N0) presenting non-mucinous 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 two 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, 26, 27). 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 Figure 1A-B and data not shown). The collection included eight 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 CRC and then after relapse with liver metastasis. This patient is the only rectal carcinoma case in our PDX collection.

Since gene expression profiles can define a particular tumor subtype (28-30) we analyzed 33 out of the 35 established PDX using the Nanostring platform with an identifier of 292 genes (Figure 1 and Supplementary Table 1). Sample preparation failed in the two remaining PDX. We observed a perfect clustering of all mucinous separated from the non-mucinous 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 (22) (Figure 1 and Supplementary Table 2). We detected mutations frequent in colorectal tumors affecting KRAS, PIK3CA, APC, TP53 or BRAF (30). 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 rectal cancer patient.

No other correlation was observed between mutational status, gene expression clusters and histopathological characteristics. Paired genotyping was performed on 12 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 CRC patient-derived cells

We derived tumor cells from colon carcinomas surgically removed from six 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 six 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 out of 29 lymph nodes. Patient 3 (sample P3) presented a pT3N1a low-grade conventional adenocarcinoma that infiltrated serosa, adipose tissue and 1 out 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 six months starting after surgery. Patient 6 (sample P6) presented a high-grade, pT4aN2b, ulceroinfiltrative mucinous adenocarcinoma that invaded the serosa and 7 out 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 10/33 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 10/33 received FOLFOX chemotherapy for five months but relapsed 16 months after the initial surgery, developing polylobulated liver metastasis (sample P33). Patient 2 died due to surgical complications and patient 5 died five months after diagnosis of relapse. The other four patients are still alive and have not, up until now, developed distant metastases, as observed by thoraco-abdominal Computerized Tomography (CT).

1x10^6 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 due to 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 Figure 1A). 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 three out of the five 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, eight out of nine mice generated a tumor in the cecum when injected with cells derived from patient 5.

Cells derived from the mucinous adenocarcinomas of patient 1 and 5 generated metastases in the abdominal cavity (carcinomatosis), lungs and liver that were confirmed by Haematoxylin/Eosin staining and immunofluorescence for EpCAM and CK20 (Table 4, Figure 2 and Supplementary Figure 2). On the contrary, cells derived from patient 2 and 33 did not generate metastases in any of the injected mice. In the case of patient 3, one mice out of three 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 six 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 Figure 3A-B).
Testing drug response in CRC 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 due to 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 out of 4 mice), whereas oxaliplatin prevented metastases formation (4 out 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 (Figure 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 CRC 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 Figure 4). $^{18}$F-FLT presented a very high uptake by normal intestinal mucosa, especially in the colon. Additionally, $^{18}$F-FLT is highly excreted into the urine producing a high radioactivity concentration at the level of the bladder. Its high uptake by intestinal mucosa and bladder made it impossible to identify or localize the tumor. The radiotracers $^{11}$C-Choline and $^{11}$C-Methionine provided equivalent results showing intense uptake in the liver and kidneys, and very low uptake in tumoral tissue. Such a
low signal combined with the high uptake present in abdominal organs, compromised the precise delimitation of the tumoral mass that was only therefore possible in a few cases.

PET with $^{18}$F-FDG allowed us to observe tumors in all cases that presented a detectable abdominal mass by manual palpation (Supplementary Figure 4). In healthy animals, this radiotracer presents a very low uptake at the level of the abdomen. However, the urinary bladder accumulated significant levels of radioactivity. For this reason, a precise evaluation of tumors in close contact with this organ could be difficult. The signal provided by this radiotracer was heterogeneously distributed or localized in a rim region at the level of the external layer of the tumor. In contrast, the inner part of the tumor mass presented a very low uptake, even lower than that observed in non-tumoral tissues (Supplementary Figure 4). The conventional adenocarcinoma derived from patient 2 showed a higher uptake than mucinous tumors from patient 1, coinciding with their differences in cellularity (Supplementary Figure 1A and 4). Large liver metastases or carcinomatosis were detected by PET when located distantly from the primary tumor growing in the cecum wall (Figure 2A and Supplementary Figure 4B).

We then performed a longitudinal study by PET with $^{18}$F-FDG to evaluate the effect of an AKT inhibitor, API-2, on tumor growth. Mice with primary carcinomas generated from patient 1, reduced tumor growth upon long-term treatment with API-2 every second day (Figure 3B and C). At the end of the experiment tumor signal was reduced in API-2 cohort compared to vehicle treated mice. On the contrary, no significant effect on tumors derived from resistant patient 2, was observed upon API-2 treatment (Figure 3D). A non-statistically significant reduction of proliferation and increase in apoptosis was observed in the tumour xenografts of API-2-treated mice injected with cells derived from patient 1, as evaluated by histological immunofluorescence of Ki67 and cleaved caspase 3 respectively (data not shown).
DISCUSSION

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 pre-clinical models that permit the testing of the efficacy of this new generation of target-directed drugs and validation of biomarkers of response.

Historically, most pre-clinical studies have been based on cell line models \textit{in vitro} and \textit{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 anti-tumoral drugs is not representative of what actually occurs in heterogeneous human carcinomas (15). It is well accepted that intratumoral heterogeneity occurs with respect to a variety of biological, biochemical and immunological properties (31). These properties determine the ability of particular cancer cell subpopulations to emerge from the primary tumor and establish metastatic growth within distant organs (32). 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 pre-clinical mouse models based on cancer cell lines. Therefore, PDX are currently becoming the best pre-clinical 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 CRC PDX collections (7-9). Most of the laboratories that have generated similar 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 set up of robust experiments to test the activity of anti-tumoral 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 re-initiate 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’ intra-tumoral 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 CRC PDX collections indicating that less aggressive tumors have a reduced capacity to engraft in immunodeficient mice (7, 26).

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 CRC patients. The PDX generated from cultured cells also preserve pluripotency since 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. 12 original patient carcinomas and PDX pairs were genotyped presenting the same mutational pattern. Only in two 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 CRC such as KRAS, PIK3CA, BRAF or APC. The analysis of APC is clearly incomplete since 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 CRC carcinogenesis.

The genetic data generated demonstrates that our PDX models faithfully represent the main genetic characteristics of CRC patients (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 CRC patients (30). Such discrepancy could be due to 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 CRC samples (30). 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 behaviour 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 CRC tumors where 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 pre-clinical approach to test target-directed therapies that could also affect the tumor stromal component.

Since metastatic CRC 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 anti-tumoral 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 pre-clinical 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 CRC patients 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’
carcinoma. We have observed peritoneal carcinomatoses, liver and lung metastases. Lung metastasis was observed in 23% of the injected mice whereas the liver was affected in 27% of all animals. Such incidences are similar to those observed in patients presenting 10% to 20% lung and 20% to 70% liver metastasis (1).

We injected a suspension of single cells instead of attaching a piece of tumor tissue to cecum wall. Tumor tissue pieces attached to the serosal side of the cecum could present a reduced capacity to reach vascular and lymphatic systems located in the submucosa. That would explain the failure of this model to generate lung metastasis. Contrarily, cancer cell injection directly under the intestinal mucosa would facilitate their access to the abundant vasculature that feeds the cecum submucosal layer, prompting cell dissemination and distant metastasis to both the lung and liver. Mice orthotopically injected with cells derived from patient 1 developed numerous distant metastases and presented tumoral cells detached from the primary xenograft tumor in the cecum that were invading local vasculature (data not shown).

We observed that cells derived from primary mucinous adenocarcinoma of patients 1 and 5 have a high metastatic potential. It is generally recognised that mucinous tumors of the colon have a worse prognosis than non-mucinous carcinomas. The mucinous adenocarcinomas have a higher penetration rate, increased lymph node invasion, less protective lymphocyte infiltration in tumor margins, higher metastatic potential and lower 5 year survival rate (33, 34). Furthermore, also in accordance with our results, in vivo studies in experimental animals demonstrated increased tumorigenicity and metastatic potential of tumors derived from cell lines that produced large amounts of mucin (35-37).

Curiously, cells derived from the liver metastasis sample P33 did not generate distant metastases when injected into the cecum of NOD-SCID mice. It is possible that tumor cells growing in the patient's liver acquired a transient capacity to escape the primary
tumor but have lost such potential after homing in the new tissue. This transient behavior has already being observed in other metastasis models (38).

Although we observed some similarities between the evolution of PDX mouse models and the clinical progression of the corresponding patients, a large scale study will be required to prove that our orthotopic PDX mouse models completely recapitulate CRC progression in patients. It would be essential to reproduce in parallel in the mouse models equivalent adjuvant chemotherapy treatments as those received by patients. Our results with six CRC patients provide the first evidence regarding the potential usefulness of our orthotopic PDX mouse model as a preclinical approach to predict disease progression of patients or drug response and hopefully help to better tailor treatment in the coming future.

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 anti-metastatic 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 anti-tumoral activity (39, 40). 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 non-invasive 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 (41, 42). 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 CRC with patient-derived cells. Indeed, PET is used in the clinic to detect the presence of metastasis or the response to treatments in colon cancer patients (43).

PDX models open a promising avenue for precision oncology since functional assays could be performed with cells derived from colon cancer patients 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 anti-tumoral 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 like 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 colon cancer patient, better guiding therefore oncologists to select the best targeted therapy.

Conclusions

We present an improved procedure to generate CRC PDX with a high tumor take rate that has allowed the generation of a rapidly expanding collection of models for further
pre-clinical 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 CRC 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.

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Olga Torres Fellowship and H.G.P. was supported by the Miguel Servet Program,
ISCIII.

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A Metastasis model with Patient-derived Colon Cancer Cells


Table 1

Primary tumors

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<td>pT4</td>
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## Table 2

Liver metastasis

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FIGURE LEGENDS

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 (44). 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).

Figure 2. Patient-derived cells preserve metastatic potential.

(A) Representative macroscopic views into the abdominal cavity of two mice (Ms1 and Ms2) injected in the cecum with colon cancer cells derived from patient 1 (P1) or derived from patient 2 (P2). Ce, cecum xenograft; M, metastasis; C, carcinomatosis. Dashed lines delineate primary xenograft tumor grown in the cecum wall. (B) A panel of Haematoxylin/Eosin staining of the corresponding carcinomatosis, lung, and liver metastasis generated in mice injected with cancer derived from P1 and P3. 1, tumoral area; 2, necrotic area. L, Liver; T, Tumor. Dashed line delineate metastasis in liver parenchyma. Scale bar, 1mm in pictures in columns 1, 3, 5 and 200 μm in magnifications in columns 2, 4, 6. (C) Lung and liver metastasis in mice injected with cells derived from P1, were immunostained for Cytokeratin 20 (CK20, red), and Epithelial Cell Adhesion Molecule (EpCAM, red). Representative confocal pictures are shown. Scale bar, 200 μm.

Figure 3. Response of orthotopic CRC PDX to chemotherapy and API2 treatment.

(A) Upper panels: 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. Lower panels: 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 $^{18}$FDG 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 during 48 days 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.

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

Cells derived from primary colorectal tumors presented different implantation rate or tumor latency when injected subcutaneously in NOD-SCID mice. Histological differentiation grade (G1-2, well/moderate; G3-4, 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’s test or Chi-square test.

Table 2. Patients’ clinical characteristics and PDX implantation rates of liver metastases.

Cells derived from liver metastastasis presented different implantation rate or tumor latency when injected subcutaneously in NOD-SCID mice. Histological differentiation grade (G1-2, well/moderate; G3-4, 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’s test or Chi-square test.

Table 3. Clinical and histopathological features of patients with derived orthotopic mouse models.
Histological differentiation grade (G1-2, well/moderate; G3-4, poor/undifferentiated). Tumor-Node-Metastasis status. pT3-pT4, invasive tumors (pT3, subserosa; pT4a, serosa); pN0, no malignant lymph nodes; pN1, 1-3 lymph node metastasis; pN1a, metastasis in one regional lymph node; pN2b, metastasis in 7 or more regional lymph nodes. Liver met, liver metastasis; n.a., not applicable; n.d., not determinate; l.n., lymph node; ad. gland, adrenal gland.

Table 4. Incidence of xenograft implantation and metastasis in orthotopic CRC PDX.
Table indicating on left, number of cells subcutaneously injected, incidence of tumor initiation, and number of passages in NOD-SCID mice; and on right, incidence of mice with metastasis upon injection into the cecum wall of cells derived from different colon cancer patients. (F), frozen.
**FIGURE 1**

![Diagram showing the localization and mutational status of various gene mutations in different tumor types.]

### PATIENT SAMPLE LOCATION

<table>
<thead>
<tr>
<th>Patient Sample</th>
<th>Localization</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>P31</td>
<td>LM</td>
<td>MUC</td>
</tr>
<tr>
<td>P32</td>
<td>R</td>
<td>MUC</td>
</tr>
<tr>
<td>P34</td>
<td>RC</td>
<td>MUC</td>
</tr>
<tr>
<td>P20</td>
<td>P30</td>
<td>MUC</td>
</tr>
<tr>
<td>P18</td>
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<tr>
<td>P12</td>
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<td>P14</td>
<td>MUC</td>
</tr>
<tr>
<td>P5</td>
<td>P17</td>
<td>MUC</td>
</tr>
</tbody>
</table>

### LOCALIZATION

- **LM**: Primary tumor
- **R**: Right colon (RC)
- **P**: Left colon (LC)
- **T**: Rectum (R)

### TUMOR TYPE

- Primary tumor: **Red**
- Right colon (RC): **Orange**
- Left colon (LC): **Yellow**
- Rectum (R): **Blue**

### MUTATIONAL STATUS

- **Mutant**: **Red**
- **Wild type**: **Blue**
- **Not analyzed**: **Gray**

*Samples from the same patient*
FIGURE 2

A

B

C

Carcinomatosis

Lung metastasis

Liver metastasis

P1

P2

P3

Lung metastasis

Liver metastasis

P1

P3

P1

P2

P3

CK20

EpCAM

CK20

EpCAM
FIGURE 3

A

Proliferation

Apoptosis

Proliferation

Apoptosis

VEHICLE

OXALIPLATIN

B

C

D

VEHICLE

API-2

VEHICLE

API-2

Tumor volume (r. u.)

Time (days)

Tumor volume (r. u.)

P = 0.0211
Clinical Cancer Research

A Personalized Pre-clinical Model to Evaluate the Metastatic Potential of Patient-derived Colon Cancer Initiating Cells

Isabel Puig, Irene Chicote, Stephan Paul Tenbaum, et al.

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