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CReMEC initiative: Establishment and characterization of new *in vivo* models of human colorectal cancers.

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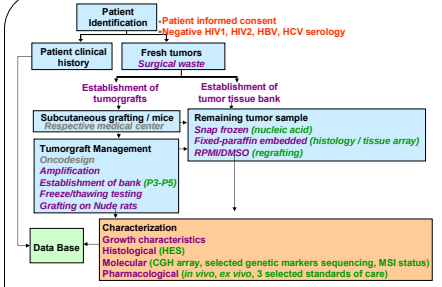
INTRODUCTION - OBJECTIVES

New well characterized models representing the heterogeneity of human colorectal cancers (CRC) are needed to develop effective therapeutic agents for that indication; establishment of such tools will allow a better prediction of the clinical outcome, taking into account the diversity of each patient tumor phenotype and genotype. For this purpose we have associated efforts from hospitals, academic groups, biotech and private pharmaceutical companies. The goal of this consortium is to create an experimental tumor model resource center to improve or strengthen drug development. We report here our results on our fully characterized CRIC004M human colon tumor model.

METHODOLOGY



- Patients were informed and gave their consent for providing surgical tumor samples to CReMEC and for HIV1, HIV2, HBC and HCV serological status testing.
- Tumor samples were collected in 3 medical centers of the Paris-Ile-de-France area: Institut Curie, Institut Gustave Roussy, Hôpital Lariboisière.
- Fresh tumor material were conditioned into RPMI 1640 with 200 U/mL penicillin, 200 µg/mL streptomycin and 2.5 µg/mL fungizone.
- Initial xenografting was carried out at the site of sampling, within 12 hours after specimen collection. Procedures were performed according to ethical guidelines for animal care and handling.
- 20-40 mg fragments were xenografted subcutaneously either in the flank or in the interscapular area, in 3-5 immunodeficient SWISS *Nude* mice
- Grafted mice were kept for a maximum of 4 months without tumor growth.

Clinical data collection

- Relevant clinical information are collected by the attending physician and included in a standardized data sheet. Identification of the data sheet is anonymous.

Histological characterization

- Samples were fixed for a maximum of 48 hours in alcohol-formalin-acetic acid (AFA) and embedded in paraffin. 5 µm sections were stained with hematoxylin-eosin-safran.

Molecular characterization - sequencing

- *CGH array analysis:* Evaluation of genome-wide, gene copy number was evaluated using a 244k CGH array Agilent technology. It was carried out on DNA from the patient sample (P0) or at an early passage (P1), and at the passage used for the pharmacological evaluation (P8-9).
- *DNA sequencing:* The following genetic markers, relevant in CRC, were selected for sequencing: *APC* (exons 9 & 16), *KRAS* (exons 2 & 3), *BRAF* (exons 11 & 15), *TP53* (exons 2 to 11), *CTNNB1* (exon 3), *PIK3CA* (exons 10 to 21), and *FBXW7* (exons 3 to 10). Sanger direct sequencing was performed after PCR amplification of exons of genes harboring hotspot mutations as described in Catalogue Of Somatic Mutations In Cancer (COSMIC: http://www.sanger.ac.uk/genetics/CGP/cosmic/).
- *Determination of Microsatellite Instability (MSI) status:* The MSI status was determined according to the Conference Consensus (or Revised Bethesda Guidelines) (*J. Natl Cancer Inst* 2004, 96, 261-268) recommendations using the following five quasimonomorphic markers: *NR21, BAT26, BAR25, NR24* and *NR22.*

Pharmacological characterization

- *Tested agents:* 5-fluorouracil (5-FU), oxaliplatin (L-OHP), irinotecan (CPT-11) or SN-38 for *ex vivo* evaluation, were tested.
- *3D ex vivo assay:* Two to 4 mm³ tumor fragments were incubated in poly (2-hydroxy ethyl methacrylate)-coated plates for 24h with either DMA vehicle control, 5-FU 1µM, SN-38 1µM or L-OHP 1µM. After 3 days of release without drugs, tumor fragment were paraffin wax embedded and proceeded for Ki67 and M30 immunohistochemistry. Number of Ki67- and M30-positive cells was evaluated to estimate drug effect on proliferation and apoptosis, respectively.
- *In vivo determination of antitumor activity:* Tumor fragments were subcutaneously xenografted in *SCID* mice. Tumor-bearing mice were randomized when the mean tumor volume reached 100-200 mm³ in mice. Compounds were formulated in glucose 5% in water, administered IV, and tested at 70% of their respective highest non toxic dose and using the following regimen: 5-FU at 56 mg/kg/adm once daily, twice, 4 days apart, L-OHP at 5 mg/kg/adm once daily, twice, 4 days apart and CPT-11 at 30 mg/kg/adm, once daily, thrice, 2 days apart. Antitumor activity was evaluated by calculating the ΔT/C *ratio:*

$$\Delta T/C\ (\%) = \frac{\text{median } T_{VOL\text{-}DY} - \text{median } T_{VOL\text{-}DX}}{\text{median } C_{VOL\text{-}DY} - \text{median } C_{VOL\text{-}DX}} \times 100$$

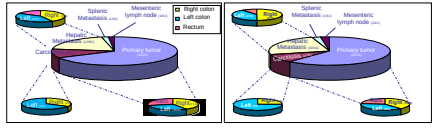
Where:
- ΔT/C ≤42 % : Moderate required to declare activity
- ΔT/C <10 % : Marked antitumor activity

RESULTS

Tumor collection

	Primary	Metastasis	Carcinosis	Total
Collected	58	20	9	87
Established in Nude mice	31	16	4	51
Take Rate	*53%*	*80%*	*44%*	*58%*

From May 2007 to December 2008, 87 surgical specimens were collected from CRC patients, and grafted in immunodeficient mice, with an overall take rate > 50%. Preliminary molecular characterizations indicate a 32% *KRAS* mutation rate on clinical samples, a mutation rate in accordance with literature data.



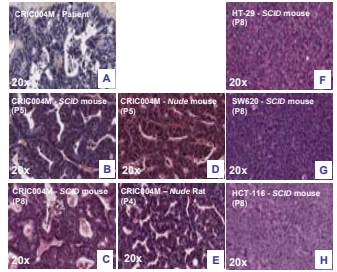
The diversity of primary tumor sampling is maintained, in the xenograft, with similar proportions

CRIC004M: The 1st characterized model

Clinical data

CRIC004M sample was obtained from a liver metastasis, synchronous of a well differentiated Lieberkühnien adenocarcinoma of the left colon of a 70 year old man. His liver metastasis was resected 4 months after left hemicolectomy and 6 cycles of FOLFIRI (folinic acid, fluorouracil, irinotecan). After liver surgery, he received 6 adjuvant cycles of FOLFIRI. He is still alive without disease (follow-up of 18 months).

Histological characterization

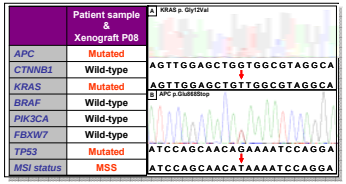


- Histological evaluation reveals a well differentiated adenocarcinoma of the colon, characterized by the presence of glandular structures.
- There is a high degree of similarity between xenografts (B, C, D, & E) and patient tumor sample (A). This is in contrast with the histological findings observed in xenograft obtained from commercially available, immortalized colon tumor cell lines (F, G, & H)
- There is a high degree of similarity in histological findings regardless of the *in vivo* passages (B & C).
- There is a high degree of similarity in histological findings regardless of the mouse strains (*SCID vs Nude* ; B & C) or the animal species (mouse *vs* rat; B, D & E).

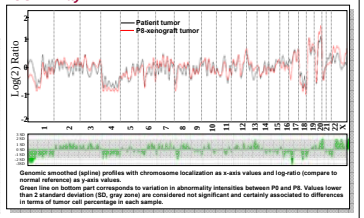
Molecular characterization

DNA sequencing and MSI status determination

	Patient sample & Xenograft P08
APC	Mutated
CTNNB1	Wild-type
KRAS	Mutated
BRAF	Wild-type
PIK3CA	Wild-type
FBXW7	Wild-type
TP53	Mutated
MSI status	MSS



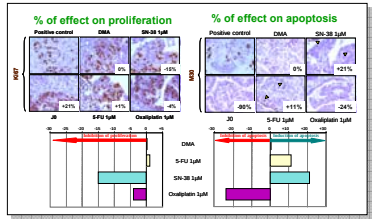
CGH Array



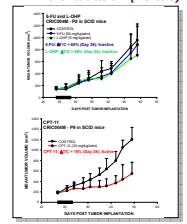
- CRIC004M is mutated:
  - in exon 16 of *APC* (B). The mutation is associated with a stabilization of β-catenin in the cytoplasm *(data not shown).*
  - in exon 2 of *KRAS* (A).
  - In exon 8 of *TP53*. The mutation is a rare missense mutation *(leucin →prolin in codon 289)*
- Several frequent polymorphisms were observed in tested genes *(data not shown).*
- CRIC004M is microsatellite stable.
- No major deviation of the genomic profile was demonstrated between patient tumor sample and xenograft model after 8 passages. We noticed only few variations in chromosomes 1 and 17.

Pharmacological characterization

3D ex vivo evaluation (P5)



In vivo evaluation (P8 & 9)



- When evaluated *ex vivo*, only SN-38 induced significant apoptosis and inhibition of tumor cell proliferation on CRIC004M fragments.
- When evaluated *in vivo*, only CPT-11 showed moderate antitumor activity in *SCID* mice at tested dose. Same pharmacological profil was obtained in *Nude* rats *(data not shown)*

CONCLUSIONS

- Over a 1.5 year period, the CReMEC consortium was able to graft 87 CRC primary tumor samples into immunodeficient mice, with a take rate >50%. All established models are under characterization with the same process as described in this poster.
- The diversity of the CRC xenografts established is similar to that of the patient tumor samples collected, indicating good representation of the initial population. The preliminary *KRAS* mutation rate evaluation of 32% is in accordance with that of the literature for colon cancer.
- Characterization of the 1st established model CRIC004M indicate the following:
  - ❑ Histological glandular structures are maintained after engraftment into animals.
  - ❑ Histological & molecular characterization indicate that the model is stable over at least 8 passages.
  - ❑ Molecular characterization suggest a Group 3 classification of CRIC004M based on Jass *et al.* *(Histopathology 2007)*
  - ❑ Initial pharmacological evaluation suggests correlation between *ex vivo* and *in vivo* drug response.
- Evaluation on additional models are needed to establish the relevance of such models for the development of anticancer drugs.

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