Evaluating patient-derived colorectal cancer-xenografts as preclinical models by comparison with patient clinical data

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Running title: IGF2-PI3K and ERBB-RAS alterations in colorectal tumorgrafts

Precis: This study highlights the translational relevancy of patient-derived colorectal cancer-xenografts which recapitulate IGF2-PI3K and ERBB-RAS genomic alteration profiles, and cetuximab response observed in patients.
**Key words:** colorectal cancer, patient-derived xenografts, genomic alteration patterns, targeted therapies

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ABSTRACT

Development of targeted therapeutics required translationally-relevant preclinical models with well characterized cancer genome alterations. Here, by studying 52 colorectal patient-derived tumor xenografts (PDX), we examined key molecular alterations of the IGF2-PI3K and ERBB-RAS pathways and response to cetuximab. PDX molecular data was compared to that published for patient colorectal tumors in The Cancer Genome Atlas. We demonstrated a significant pattern of mutual exclusivity of genomic abnormalities in the IGF2-PI3K and ERBB-RAS pathways. The genomic anomaly frequencies observed in microsatellite stable PDX reproduce those detected in non-hypermutated patient tumors. We found frequent IGF2 upregulation (16%), which was mutually exclusive with IRS2, PIK3CA, PTEN and INPP4B alterations, supporting IGF2 as a potential drug target. In addition to maintaining the genomic and histological diversity, correct preclinical models need to reproduce drug response observed in patients. Responses of PDXs to cetuximab recapitulate also clinical data in patients, with partial or complete response in 15% (8/52) of PDXs and response strictly restricted to KRAS wild type models. The response rate reaches 53% (8/15) when KRAS, BRAF and NRAS mutations are concomitantly excluded, proving a functional cross validation of predictive biomarkers obtained retrospectively in patients. Collectively, these results show that, because of their clinical relevance, colorectal PDXs are appropriate tools to identify both new targets, like IGF2, and predictive biomarkers of response/resistance to targeted therapies.
INTRODUCTION

Colorectal cancer (CRC) remains a major cause of mortality worldwide and CRC patient death is generally attributable to metastasis development. Comprehensive molecular characterization of CRC has identified key gene and pathway alterations important for initiation and progression of CRC, including alterations in the PI3K and ERBB-RAS pathways (1, 2). Some genetic anomalies have been also shown to predict response to specific therapies, such as activating mutations in \textit{KRAS} which predict resistance to anti-EGFR monoclonal antibodies (MAb) (3). For efficient development of new therapies and companion biomarkers, preclinical models mimicking the molecular epidemiology and drug sensitivity of human tumors are needed.

In CRC, tumor-specific patient-derived xenograft (PDX) models have shown to retain the intratumoral clonal heterogeneity, chromosomal instability, and histology of the parent tumor through passages in mice (4-7). To extend these observations, we investigated here a collection of 52 colorectal PDXs (6), composed of 48 MSS (microsatellite stability) and 4 MSI (microsatellite instability) tumors, for the presence and prevalence of molecular features reported in large CRC patient cohorts (1, 2, 8). In particular, we studied key alterations in IGF2-PI3K and ERBB-RAS pathways and the role of these alterations in predicting response to cetuximab.
MATERIALS and METHODS

Patient-derived tumor xenografts. Tumor xenografts were established directly from patient tumors (6) and were routinely passaged by subcutaneous engraftment in immunodeficient CB17-SCID mice (Charles River Laboratories). Xenografts, passage P6-P9, were harvested from 3 mice for each model, when they reached around 150-300 mm$^3$ in size for RNA and DNA extraction. For in vivo pharmacological studies, Cetuximab (Imclone) was given at 12.5 mg/kg/adm, (Q3Dx2)x2 IP), mice bearing 100-200 mm$^3$ tumors at start of therapy (n = 8-10 per group) as already described (6). All experimental procedures were approved by Sanofi Laboratory Animal Care and Use committee.

Microsatellite Instability (MSI) status. MSI testing was performed according to the National Cancer Institute guidelines using a five-microsatellite consensus panel (6).

DNA sequencing. Next-generation sequencing and mutation calling were performed at Beijing Genomics Institute (BGI). Library preparation was performed using exome capture Agilent SureSelect All Exon 50M. Libraries were sequenced using the Illumina HiSeq platform. Quality single-nucleotide polymorphism (SNP) calling criteria have been applied: SNP quality is equal or greater than 20; the minimum sequencing depth is 4X and the mean is 100X, with 99% of coverage target region. To evaluate and eliminate the false positive SNPs calls generated by cross hybridization with mouse DNA, we have detected and filtered out reads aligned to mouse reference sequences before doing human whole-exome sequencing analysis; by this way, only specific human calling are considerate.. Besides, KRAS, BRAF and PIK3CA mutations were validated by Sanger method in a different tumor sample.

CGH array analysis: Evaluation of genome-wide, gene copy number was performed using the 250k and 400k oligonucleotide CGH array Agilent technology using two biological
duplicates and two independent experiments. Oligonucleotide array CGH processing was performed as detailed in the manufacturer's protocol (version 6.2 October 2009; http://www.agilent.com). The log2 ratio and segmentation were generated using Array Studio software. Array Studio, Array Viewer, Array Server and all other Omicsoft products or service names are registered trademarks or trademarks of Omicsoft Corporation, Research Triangle Park, NC, USA.

**Gene expression profiling.** The analysis of gene expression was done using U133 Plus Affymetrix microarrays with biological triplicate (3 tumor tissues removed from 3 distinct mice for each model, passage P6-P9).

**Real-time RT-PCR.** Affymetrix data of candidate genes were confirmed by qRT-PCR using previously described methodology (9).

**Immunohistochemistry.** PTEN and INPP4B expression were determined on 4-μm-thick AFA-fixed paraffin-embedded sections. Antigen retrieval was done by incubating tissue sections in an 850 W microwave oven for 36 min in Tris-EDTA or in citrate buffer for INPP4B and PTEN staining, respectively. Tissue sections were then incubated for 1 hour at room temperature with primary antibodies (anti-INPP4B, clone EPR3108Y, dilution 1/50, rabbit mAb, LSBio; anti-PTEN, clone SP218, dilution 1/50, rabbit mAb, Spring). Staining was revealed by using OmniMap HRP anti-Rabbit (Ventana Medical system) and diaminobenzidine (DAKO) as chromogen.
RESULTS and DISCUSSION

Comprehensive molecular characterization of tumor samples from CRC patients has identified a handful of recurrent mutated genes within critical pathways (1, 2, 10). Among these, the PI3K and ERBB-RAS signaling, accurately dissected by the Cancer Genome Atlas Network (TCGA) (2), provide promising therapeutic targets.

In order to gain more insight into the genomic abnormalities within the PI3K and ERBB-RAS signaling pathways, a large cohort of 52 colorectal PDXs established by the CReMEC consortium (6) (48 MSS and 4 MSI tumors) was analyzed.

We first examined 6 genes identified as key upstream elements in the PI3K pathway (2): IGF2, IRS2, PIK3CA, PIK3R1, PTEN and INPP4B (Figure 1A, Table 1). Several lines of evidence underline the importance of IGF2 in colorectal cancer. IGF2 is the single most overexpressed gene in colorectal neoplasia relative to normal colorectal mucosa (11) and loss of imprinting of IGF2, one mechanism for its frequent overexpression, is also a risk factor for CRC (12). More recently, TCGA revealed IGF2 as an important node in the PI3K pathway with mutual exclusion between IGF2, IRS2, PIK3CA, PIK3R1 and PTEN genomic alterations. Here, gene expression analyses identified IGF2 overexpression in 7 PDXs. As reported in patients (1, 2), IGF2 overexpression in PDXs (5 out of 7) is mainly due to focal IGF2 amplification (Figure 1A-B).

The binding of IGF2 to IGF1R activates the intrinsic tyrosine kinase activity of IGF1R, which results in the phosphorylation of the IRSs (Insulin Receptor Substrates), leading to PI3K activation. Gene expression analysis of IRS1 and IRS2 revealed no alterations in IRS1. However, overexpression of IRS2 (n=5) was detected in mutually exclusive pattern with IGF2 amplification or overexpression (Figure 1A). All PI3KCA aberrations (n=12) were oncogenic mutations, affecting all functional domains of the enzyme but with preferential mutation hotspots within exons 9 and 20, as previously described in CRC (13). PIK3R1 mutations have been rarely reported in CRC (2) and none were detected in the present PDX collection. Two PDXs showed PTEN homozygous deletion associated with loss of protein expression...
while no PTEN mutation was detected (Figure 1C). Recently, another lipid phosphatase, Inositol Polyphosphate 4-phosphatase type II (INPP4B), has emerged as a potential tumor suppressor in prostate, breast, and ovarian cancers (14). Downregulation of INPP4B gene expression was detected here in 2 PDXs, with concomitant loss of protein expression. Immunohistochemical analyses confirmed mutual exclusion between PTEN and INPP4B downexpression (Figure 1C).

Interestingly, a pattern of mutual exclusion in the PI3K pathway also exists between IGF2, IRS2, PIK3CA, PTEN and INPP4B alterations. These data imply that therapeutic targeting of the IGF2 pathway could inhibit PI3K activity and suggest INPP4B as a tumor suppressor gene in CRC.

Mutations or gene amplification of candidate genes in the ERBB-RAS pathway was then analysed. EGFR displayed no mutations but gene amplification associated with gene overexpression in 2 MSS PDXs. No mutation was identified in ERBB2, but one PDX showed ERBB2 amplification, accompanied by overexpression. Two PDXs displayed a T389I ERBB3 mutation, probably damaging (PolyPhen® prediction software). No gene alteration was present in ERBB4.

We found that 69% (33/48) of MSS tumors and 100% (4/4) of MSI tumors have oncogenic alterations in KRAS, NRAS or BRAF with a significant pattern of mutual exclusion (Figure 1A). In accordance with published data, KRAS missense mutations in codons 12, 13 and 61 were the most frequent KRAS mutations (observed mutated in 18, 5 and 2 PDX models, respectively). Two additional PDXs showed 2 oncogenic KRAS mutations, K117N and A146T, previously reported in CRC with similar low frequencies (1, 15). Six PDXs displayed NRAS mutations, with all mutations occurring in codon 61. Six PDXs displayed BRAF mutations: Four of these were the frequent hot-spot V600E mutation and two were less frequent mutations, D594N and G469A, already reported in CRC (16). BRAF V600E mutations were associated with microsatellite instability, as this mutation was present in 75%
(3/4) of MSI tumors compared to 2% (1/48) of MSS tumors, \( p < 0.0001 \), Yates' \( \chi^2 \) test. \( BRAF \) V600E mutations were mutually exclusive from \( KRAS \) and \( NRAS \) mutations as usually described (16).

Finally, we observed no significant association of alterations in the RAS and PI3K pathways, suggesting that simultaneous inhibition of the RAS and PI3K pathways might be necessary for successful therapy in the subgroup displaying co-occurrence of these molecular alterations.

These genomic analyses enable an assessment of the diversity and the frequency of genomic changes altering these two major signaling pathways in our CRC PDX models and comparison with TCGA data (Figure 2). TCGA has reported that 77% (23/30) of hypermutated tumors are MSI tumors (2). As the present PDX collection displays a low frequency of MSI tumors (4/52, 8%) close to that of patient tumors (23/224, 10%, (2)), we focused on MSS PDX and patient tumors. The frequency of studied molecular epidemiology data from these 2 groups showed remarkable concurrence, suggesting that the PDX bank represents a useful set of preclinical models for testing new therapies and emphasizing the potential therapeutic value of targeting IGF2 in CRC. In the same way, recent analyses by the Bodmer laboratory have shown that \( in vitro \) CRC cell lines provide useful preclinical tools because of well represented genetic diversity of patient tumors in cell lines (17, 18). It led us to an analysis of gene abnormalities specifically within IGF2-PI3K pathway in a large panel of 62 human colorectal cancer cell lines using the Broad-Novartis Cancer Cell Line Encyclopedia data (http://www.broadinstitute.org/ccle/home). We carefully separated MSS and MSI cell lines because of overrepresentation of MSI cell lines which could interfere with mutation frequencies. IGF2-PI3K pathway alterations appear almost mutually exclusive within the 36 MSS cell lines with some redundancy between \( PIK3CA \) activating mutations and \( IRS2 \) overexpression (Supplementary Figure 1). While \( PIK3CA, PTEN \) and \( PIK3R1 \) aberration profiles recapitulate the patient tumour observation, the frequencies of \( IGF2 \) and \( IRS2 \) upregulation in MSS colorectal cancer cell lines are under- and over-represented,
respectively.

In addition to maintaining the genomic and histological heterogeneity, translationally-relevant preclinical models need to reproduce drug response observed in patients. While KRAS mutations had been identified as a strong predictive biomarker of resistance to cetuximab and panitumumab (3), only a subset of KRAS wild-type (WT) patients respond to anti-EGFR MAbs, underlining that additional predictive biomarkers exist within KRAS WT tumors. The characterization of alterations occurring in additional candidate genes (NRAS, BRAF, PIK3CA, PTEN) increased indeed the negative predictive value up to 70%, but it is not sufficient to identify all resistant cases (19).

To assess drug response prediction in our PDX models, cetuximab response was analysed in the PDX panel. To be consistent with clinical criteria, we considered responders the PDXs displaying partial or complete response and non-responders the PDXs displaying growth stabilization or progression (Supplementary Figure 2). With these scoring criteria, 8 out of 52 PDXs (15%) were responders to cetuximab, with complete tumor disappearance in 3 PDX models. The all 8 responders were WT KRAS tumors (Figures 1A and 3), outlining the requirement of WT KRAS genotype for clinical benefit. This low proportion of PDX responders in an unselected population (15%) is highly concordant with patient data (8) and PDX data from an independent metastatic colorectal cancer xenograft series (7). Noteworthy, cetuximab had been shown to be active against KRAS mutated xenografts (6) leading to a statistically significant reduced tumor growth but with no tumor shrinkage. This kind of PDX response means nevertheless progressive disease from a clinical point of view. Therefore, assessment parameters have to be carefully analysed to avoid over- or misinterpretation of drug efficacy.

The majority of non- responder PDXs (27/44) showed canonical activating mutations in KRAS (codons 12, 13, 61, 117 and 146). The Trusolino group reported similar results involving KRAS mutations in codons 61, 117 and 146 in primary resistance to cetuximab in a large and independent colorectal PDX series (7). While most clinical studies limited KRAS
mutation assessment to codons 12–13, \textit{KRAS} codon 61 and 146 mutations, in addition to \textit{NRAS} and \textit{BRAF} mutations, have also been shown in retrospective studies to predict resistance to cetuximab or panitumumab in WT \textit{KRAS} codon 12 and 13 metastatic CRC (19, 20). The European Medicines Agency recently updates and restricts the indication for cetuximab to WT \textit{RAS} metastatic colorectal cancer (not only WT \textit{KRAS} codon 12-13).

Likewise, none of the 4 \textit{BRAF} V600E-mutated, 6 \textit{NRAS}-mutated and 4 \textit{KRAS} (codon 61, 117 or 146) mutated PDXs responded to cetuximab with tumor shrinkage. Therefore, exclusion of these mutations enables an improved selection of PDXs likely to respond to cetuximab, increasing the response rate from 28\% (8 out of 29 12-13 codons \textit{KRAS} WT) to 53\% (8/15) in PDXs that are fully wild-type for all three genes, as reported retrospectively in patients (19, 20). This study functionally cross validates a recent clinical stratification based on combination of predictive biomarkers obtained retrospectively in patients (19). These data support the utility of our PDX panel for identifying predictors of drug response in metastatic colorectal cancer patients.

As for \textit{PTEN} and \textit{PIK3CA} impact, clinical data are more conflicting (8, 13, 19). In the present preclinical work, only \textit{PTEN} homozygous deletion, leading to absolute \textit{PTEN} inactivation, has been taken into account. This \textit{PTEN} loss occurred within \textit{KRAS} mutated xenografts, displaying lack of response to cetuximab. Individual contribution of \textit{PIK3CA} mutations to the absence of response is difficult to assess because of the \textit{PIK3CA} mutation diversity in different protein domains and coexistence of these mutations with \textit{KRAS} and \textit{BRAF} mutations (13). Moreover, 1 PDX with \textit{IGF2} activation and 2 others PDXs with \textit{PIK3CA} mutation respond to cetuximab. Among the 5 \textit{IGF2}-overexpressed \textit{KRAS} WT tumors, only one responds to cetuximab. Taken together, these data suggest that IGF2-PI3K components are not biomarkers of resistance to anti-EGFR therapies and underline the interest to combine anti-IGF2 and anti-EGFR treatment.

Within the group of 15 \textit{KRAS/BRAF/NRAS} wild-type PDXs, further investigation has been performed for additional putative predictive biomarkers of resistance to anti-EGFR (8, 21): \textit{EGFR} gene amplification, overexpression and mutation; gene overexpression of two
EGFR-ligands (epiregulin and amphiregulin), MET gene amplification and overexpression, KRAS gene amplification and HRAS mutation (Supplementary Table 1). Noteworthy, none of the patients with colorectal cancer, from whom the triple wild-type PDXs were derived, had been exposed to anti-EGFR therapy prior to surgery, ruling out the possibility of acquired resistance in the pre-treated PDXs. The analysis of these parameters did not allow to statistically discriminating between the responder and non-responder groups (Fisher’s exact test, p>0.05). Nevertheless, it is noteworthy that cetuximab treatment was ineffective in mice engrafted with the 3 PDX models carrying KRAS amplification.

Collectively, the present data demonstrate the relevance of colorectal PDXs as models for preclinical drug development. The PDX models remarkably fit the molecular epidemiology and the cetuximab drug response profiles of CRC patient populations, justifying the growing use of mouse clinical trials in cancer drug development and decision making (5). More importantly, these data support the identification of KRAS (exon 2, 3 and 4) / NRAS / BRAF wild-type patients for treatment with cetuximab, and IGF2 as an attractive novel cancer drug target in a large subset of CRC patients.

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Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. *Cancer Res* 2014;74:3238-47.


LEGENDS

Figure 1. Molecular alterations of the IGF2-PI3K and ERBB-RAS pathways in MSS and MSI colorectal cancer xenografts. A, Genomic alterations of the IGF2-PI3K and ERBB-RAS pathways. Mutations were determined by NGS. Tumors were considered to be amplified if the gene copy number was >3 using CGH array analysis. Gene overexpression is defined by an expression superior to average in all PDX panel + 1 SD. B, Correlation of expression levels with copy-number changes for IGF2. Amplification: >3 gene copy; Focal amplification: <10 genes. C, In situ expression of PTEN and INPP4B proteins. Anti-PTEN and INPP4B immunohistochemistry results for representative negative (blue staining) and positive (brown staining) PDXs. Magnification x40.

Figure 2. Diversity and frequency of genetic changes leading to deregulation of IGF2--PI3K and ERBB-RAS signalling pathways in the CRC patient derived xenograft panel compared to published human tumors. MSS xenografts were analyzed for somatic mutations (ERBB2, ERBB3, KRAS, NRAS, BRAF V600E, PIK3CA, PIK3R1), homozygous deletion (PTEN), amplifications (IGF2, ERBB2), and significant gene overexpression (IGF2, IRS2). Alteration frequencies are expressed as a percentage of the 50 MSS xenografts in blue. Data obtained in non-hypermutated patient tumor samples reported by the The Cancer Genome Atlas (2) are noted in red.

Figure 3. Graphic representation of the cohort of 52 colorectal xenografts treated with cetuximab. Molecular alterations mutually exclusive or coexisting are indicated according to different color codes.
<table>
<thead>
<tr>
<th>Tumor patient information</th>
<th>Gene expression</th>
<th>Gene copy number</th>
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Table 1. The 52 PDXs have been analyzed for gene expression and gene copy number. Gene expression value measured by qRT-PCR is expressed as normalized expression. For each gene, only probe sets specific for gene transcript sequences have been analysed. *NA, not applicable*
**Figure 1.**

### Gene Expression

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### Assessments

- **Non responder**
- **RESPONDER**

### Gene Overexpression

- Gene overexpression
- Gene overexpression with gene amplification
- Gene downexpression

### Gene Mutations

- Homozygous deletion
- Classical activating mutation
- Rare activating mutation

### mRNA Expression

![Graph showing mRNA expression levels](image-url)
Figure 2.

IGF2-PI3K signaling

ERBB2/3-Ras signaling

IGF2
16% / 22%

IGF1R

IRS2
6% / 7%

PTEN
4% / 4%

PIK3CA
23% / 15%

PIK3R1
0% / 2%

ERBB2
2% / 6%

ERBB3
4% / 4%

NRAS
13% / 10%

KRAS
54% / 43%

BRAF
2% / 3%
**Figure 3.**

The diagram illustrates the classification of responders and non-responders based on the status of KRas, BRAF, and NRAS.

- **Responders**
  - KRas* Codons 12 & 13
  - Other KRas mutations
  - KRas Wild type

- **Non-responders**
  - KRAS mutated
  - BRAF V600E
  - NRAS mutated
  - KRAS/BRAF/NRAS wild-type

Legend:
- Red: KRAS mutated
- Green: BRAF V600E
- Blue: NRAS mutated
- Black: KRAS/BRAF/NRAS wild-type
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