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KRAS and *PIK3CA* mutation frequencies in patient derived xenograft (PDX) models of pancreatic and colorectal cancer are reflective of patient tumors and stable across passages

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Introduction

One key obstacle to the translation of advances in cancer research into the clinic is a deficiency of adequate preclinical models that recapitulate human disease. The development and application of validated preclinical models that reflect patient histological, cellular, and molecular characteristics is needed. Current preclinical models rely heavily on conventional cell line xenograft models which are established by engrafting human tumor cell lines cultured in the laboratory into mice. This model is widely acknowledged to provide useful, but unreliable predictive capacity for anti-tumor activity in humans (Sharpless and Depinho 2006). One possible explanation for the unreliability of cell line xenograft results translating to the clinic, is that these cells represent clonal tumor populations that have selectively grown on plastic and have adapted to growth outside of the natural tumor microenvironment (Frese and Tuveson 2007; Tentler et al. 2012). Because cell line xenograft models lack stromal cells, which are increasingly recognized as a critical element for tumorigenesis, these models fail to accurately recapitulate tumor biology and tumor response to therapy (Bhowmick et al. 2004; Sharpless and Depinho 2006; Frese and Tuveson 2007).

To overcome these disadvantages patient-derived xenografts (PDX), which are established by engrafting fresh patient tumor tissue into immunocompromised mice, have been developed **(Figure 1)**. PDX models are advantageous because they capture tumor heterogeneity and architecture (Sausville and Burger 2006; Siolas and Hannon 2013). PDX models have been shown to be better predictive models for the evaluation of novel therapeutics than cell line xenografts across multiple tumor types (Tentler et al. 2012). A large retrospective review comparing preclinical PDX response rates with Phase II clinical trial response rates found that the PDX models were reliable in predicting response for non-small cell lung cancer and ovarian cancer (Voskoglou-Nomikos et al. 2003). In another study, a panel of 80 PDX (breast, lung, ovarian, testicular, and colon cancer) was shown to have a high clinical predictive value for treatment sensitivity and resistance (Fiebig et al.

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2004). Furthermore, data obtained using PDX models have already been successfully translated into the design of clinical trials (Furman et al. 1999; Hidalgo et al. 2011). Given this strong correlation there is much excitement to use PDX models for the study of novel therapies and biomarkers (Bang et al. 2013; Neel et al. 2014). These studies reinforce the vital role that PDX play in the understanding of the biology of human disease and their potential utility to translating results into clinical practice.

One key advantage of PDX models is their availability as a renewable resource. Thus multiple therapies may be simultaneously evaluated on the same PDX tumor line. Examination of PDX across multiple passages has found that histologic and gene expression profiles are retained (Siolas and Hannon 2013). Studies of early passage (fewer than three passages) PDX models of multiple solid tumors show that mutations of the source patient tumor are retained (Rubio-Viqueira et al. 2006; Fichtner et al. 2008; Sivanand et al. 2012; Zhang et al. 2013). Although many studies show overall genomic stability across passages whether specific mutations are retained in later passages has not been well studied (Julien et al. 2012; Laurent et al. 2013; Zhang et al. 2013). There is concern that selective pressure and genetic instability could lead to mutational drift over multiple passages, and thus late passage PDX could be an inaccurate reflection of patient tumors (Tentler et al. 2012). Therefore in this study we evaluated if *KRAS* and *PIK3CA* mutations were retained at late passages in primary colorectal cancer (1°C CRC), metastatic colorectal cancer (mCRC), and primary pancreatic ductal adenocarcinoma (PDAC) PDX and whether mutational frequency is reflective of patient populations.

Materials and Methods

PDX Expansion

PDAC, 1°C CRC, and mCRC tumor tissue from de-identified patients were engrafted subcutaneously into the flanks of immunocompromised mice, expanded, and passaged over time. All animal experiments were carried out under protocols approved by the University of North Carolina Institutional Animal Care and Use Committee.

DNA Isolation

Tumors were harvested and flash frozen. DNA was isolated using the AllPrep Kit (Qiagen).

Mutational analysis of *KRAS* by pyrosequencing

Polymerase chain reaction (PCR) of exon 2 to detect *KRAS* codon 12 and 13 mutations was performed using the following primers: 5’ – CGATGGAGGAGTTTGTAAATGAA – 3’ and 5’ - /BioTEG/TTCGTCCACAAAATGATTCTGA – 3’. PCR amplification was done for 55 cycles with an annealing temperature of 58 C. PCR products were analyzed using pyrosequencing with the Pyromark MD (Qiagen) using the internal primer 5’ – AAACTTGTGGTAGTTGGA – 3’.

Mutational analysis of *PIK3CA* by pyrosequencing

PCR of exon 9 to detect *PIK3CA* codon 542 and 545 mutations was performed using the following primers: 5’ – CCATTTTAGCACTTACCTGTGAC – 3’ and 5’ - /BioTEG/

ATTTCTACACGAGATCCTCTCTCT – 3'. PCR amplification was done for 55 cycles with an annealing temperature of 62 C. PCR products were analyzed with pyrosequencing using the internal primer 5' – TTCTCCTGCTCAGTGAT – 3' for codon 542 and the internal primer 5' – TAGAAAATCTTTCTCCTG – 3' for codon 545. PCR of exon 20 to detect *PIK3CA* codon 1047 mutations was performed using the following primers: 5' – TGAGCAAGAGGCTTTGGAGTAT – 3' and 5' - /BioTEG/ TGCTGTTTAATTGTGTGGAAGATC – 3'. PCR amplification was done for 55 cycles with an annealing temperature of 62 C. PCR products were analyzed with pyrosequencing using the internal primer 5' – GAAACAAATGAATGATGC – 3'.

Results *KRAS* mutations in PDAC and CRC PDXs

We examined mutations in *KRAS* codons 12 and 13 in 30 PDAC, 32 mCRC and five 1°C CRC PDX. We found that the frequency of *KRAS* mutations in PDAC PDX was 77% (23 of 30) **(Figure 2a).** All mutations were located at *KRAS* codon 12 and were either G12V (13 of 23 (57%)) or G12D (10 of 23 (43%)). *KRAS* mutations were found in 41% (15 of 37) of 1°C CRC and mCRC PDX **(Figure 2a)**. Mutations identified in mCRC were *KRAS* G12V (3 of 14 (21%)), G12D (4 of 14 (29%)), G12S (3 of 14 (21%)), G13D (3 of 14 (21%)), and G12A (1 of 14 (7%)). One of five (20%) 1°C CRC PDX showed a *KRAS* G12V mutation.

PIK3CA mutations in PDAC and CRC PDXs

PDX tumors were examined for mutations in *PIK3CA* codons 542, 545 and 1047. A single *PIK3CA* E545K mutation was found in 1 of 30 PDAC PDX (3%) **(Figure 2b).** *PIK3CA* mutations were found in 8% (3 of 37) of 1°C CRC and mCRC PDX **(Figure 2b).** Two of 32 (6%) mCRC PDX had E545K mutations. Both were associated with a G13D *KRAS* mutation. One mutation was identified in 1°C CRC *PIK3CA* H1047R (1 of 5 (20%)) and was not associated with a *KRAS* mutation.

KRAS and *PIK3CA* mutational status retained across passages

KRAS and *PIK3CA* mutations were evaluated at later passages **(Figures 3 and 4).** PDAC PDX passages 2 - 8 were analyzed. *KRAS* and *PIK3CA* mutational status was retained in all passages examined in PDAC PDX. 1°C CRC and mCRC PDX passages 2 - 4 were analyzed. *KRAS* and *PIK3CA* mutational status was retained across all passages evaluated in 1°C CRC and mCRC PDX.

Discussion

The aim of this study was to evaluate if the mutational frequency of key mutations, *KRAS* and *PIK3CA*, in 1°C CRC, mCRC, and PDAC PDX, remained stable across late passages and was reflective of patient populations. Our analysis demonstrated that the frequency of *KRAS* mutations in PDAC was 77%. This correlated with previous studies that have documented that the *KRAS* mutational frequency in PDAC is 71-100% (Almoguera et al. 1988; Hruban et al. 1993; Pellegata et al. 1994; Hidalgo 2010; Schultz et al. 2012). It is well known that *KRAS* is one of the key early driver mutations in PDAC (Hingorani et al. 2003; Hezel et al. 2006). Activating mutations in *KRAS* impair its intrinsic GTPase activity, thus

resulting in a protein that is constitutively active, stimulating multiple key kinase pathways integral to cellular survival and proliferation (Hidalgo 2010). This has been confirmed using genetically engineered mouse models in which an activating *KRAS* mutation was sufficient for the development of precursor pancreatic cancer lesions known as pancreatic intraepithelial neoplasia (Hingorani et al. 2003). *KRAS* mutational status in CRC has been shown to be an important predictive biomarker of resistance to anti-EGFR therapy and an early mutation in the genetics of CRC (Vogelstein et al. 1988; Amado et al. 2008; Karapetis et al. 2008; Allegra et al. 2009; Walther et al. 2009). In this study we also evaluated *KRAS* mutational frequency in CRC PDX. We demonstrated that the frequency of *KRAS* mutations in CRC was 41% which correlated with previous studies that have documented a mutational frequency of 35 - 51% (De Roock et al. 2010; Janku et al. 2011; Tan and Du 2012).

We found a frequency of *PIK3CA* mutations in PDAC PDX of 3%. This correlates with previous studies that have documented a mutational frequency of 0-11% (Janku et al. 2011). Our analysis demonstrated that the frequency of *PIK3CA* mutations in CRC PDX was 8%. This result was slightly lower than the 12-21% frequency reported previously. Samuels et al. analyzed 234 CRC tumors for *PIK3CA* mutational status (Samuels et al. 2004). The overall frequency of *PIK3CA* mutations identified was 32%. This may be explained by the fact that they evaluated all known *PIK3CA* single nucleotide polymorphisms (SNPs), whereas our analysis only evaluated the three most common sites (codons 542, 545, and 1047). Janku et al. evaluated 54 CRC tumors for *PIK3CA* mutational status and identified a 14% *PIK3CA* mutational frequency for codons 542, 545, and 1047 (Janku et al. 2011). Similarly, De Roock et al. analyzed 773 CRC tumors and identified a 12% *PIK3CA* mutational frequency for codons 542, 545, and 1047 (De Roock et al. 2010). Our results were not significantly different than those reported in Janku et al. and De Roock et al. (p = NS). While the mutational frequency of 8% that we identified is slightly lower than previously published studies our sample size is small. This result may also be reflective of the population seen in this single institution study.

Of the three *PIK3CA* mutations identified in CRC the two mutations in exon 9 were associated with *KRAS* mutations, whereas the sole exon 20 mutation identified was not. This finding is in agreement with previous reports that show associations between exon 9 of *PIK3CA* and *KRAS* mutations and not exon 20 (De Roock et al. 2010).

There is concern that genetic drift can occur over late passages in PDX (Julien et al. 2012; Tentler et al. 2012). Because of this, late passage PDX are not routinely used for preclinical drug evaluation (Tentler et al. 2012; Mattie et al. 2013). To evaluate this possibility we characterized PDX genetic stability over late passages. We found that the mutational status of *KRAS* and *PIK3CA* was 100% preserved across both early and late passages analyzed in PDAC and CRC PDX. This finding suggests that genetic profiles remain stable over late passages despite potential selection pressures and reinforces the utility of late passage PDX in preclinical experiments.

Conclusion

In conclusion, mutational frequencies in 1°C CRC, mCRC and PDAC PDX closely parallel that of patient populations and crucial mutations remain stable across late passages. The accurate mirroring and stability of genetic changes in PDX models compared to patient tumors suggest that these models are good preclinical surrogates for patient tumors.

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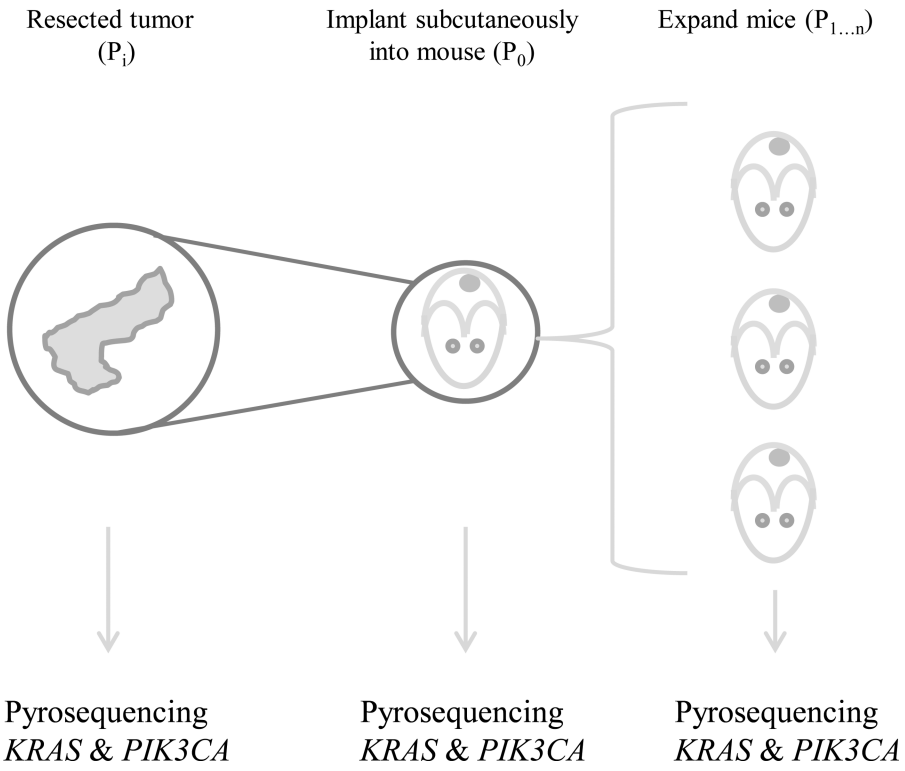

Figure 1. Establishment of patient derived xenograft mouse models

Tumor pieces (P_i) are implanted subcutaneously into immunocompromised mice (P_0). After tumors are established they are harvested, split, and passaged into additional mice ($P_{1...n}$). Tumor sections are flash frozen and DNA isolated for pyrosequencing initially and at each passage to evaluate *KRAS* and *PIK3CA* mutational status.

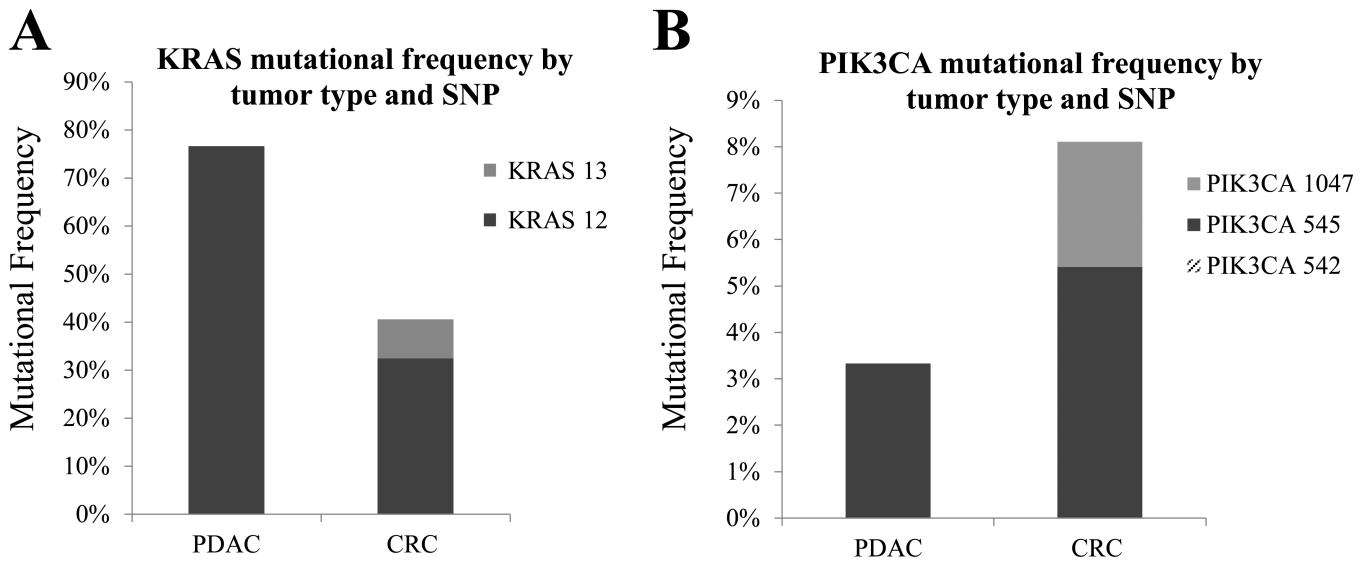

Figure 2. Frequency of *KRAS* and *PIK3CA* mutations in PDAC and CRC PDX

(A) *KRAS* codon 12 and 13 mutations in PDAC and CRC PDX (B) *PIK3CA* codon 542, 545, and 1047 mutations in PDAC and CRC PDX. (PDAC = pancreatic ductal adenocarcinoma, CRC = primary and metastatic colorectal cancer, SNP = single nucleotide polymorphism)

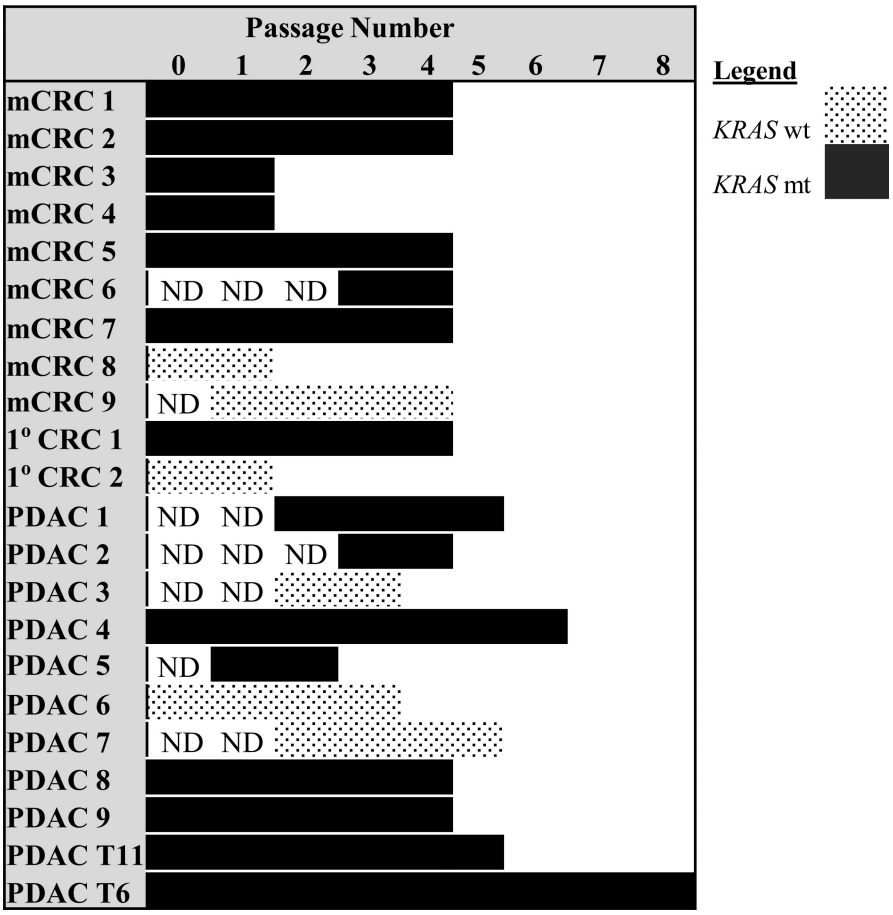

Figure 3. *KRAS* mutations are stable across passages

KRAS mutation status across passages for metastatic CRC (mCRC), primary CRC (1°C CRC), and pancreatic ductal adenocarcinoma (PDAC) PDXs. (ND = not done)

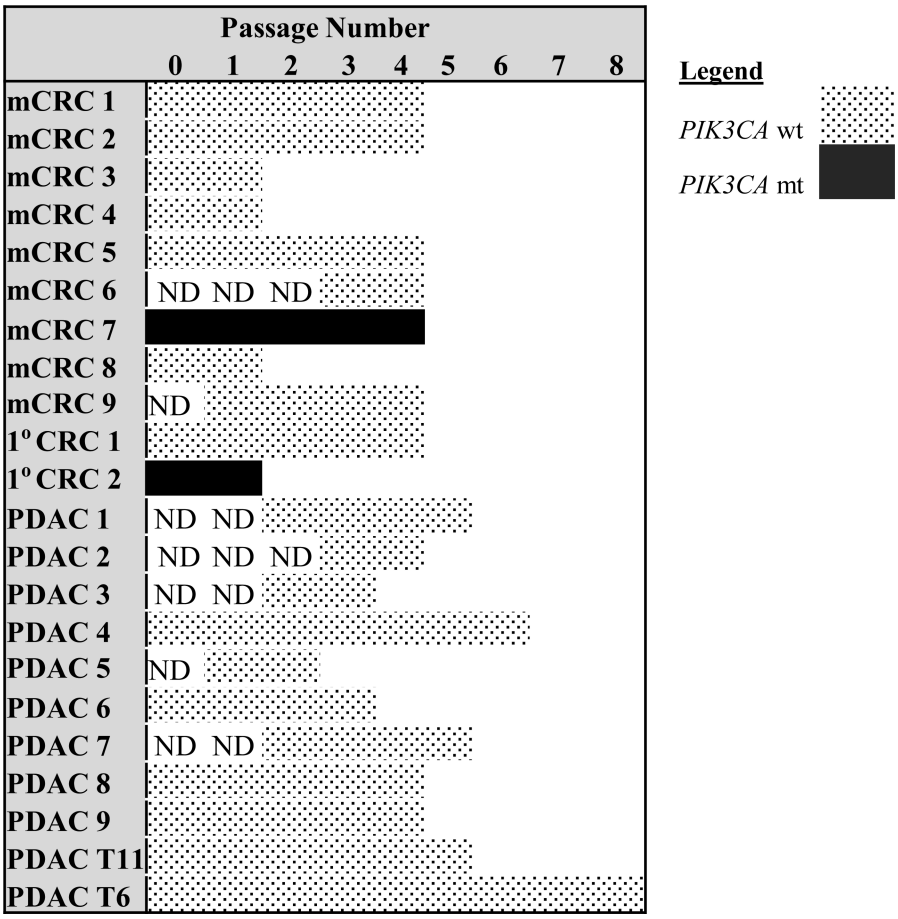

Figure 4. *PIK3CA* mutations are stable across passages

PIK3CA mutation status across passages for metastatic CRC (mCRC), primary CRC (1°C CRC), and pancreatic ductal adenocarcinoma (PDAC) PDXs. (ND = not done)