

SHORT COMMUNICATION

Identification of tumorigenic and therapeutically actionable mutations in transplantable mouse tumor cells by exome sequencing

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Cancer development occurs in response to the successive accumulation of mutations that eventually targets key regulators of cell proliferation. As most mutations likely occur randomly, cancer driver mutations can only be found if they are recurrent. Here we use exome sequencing of the mouse cell lines Panc02, L1210 and Colon 26 to identify genetic alterations (single-nucleotide polymorphisms and small insertion and deletions) that occurred in three different strains of mice and that resulted in tumorigenesis. We identify known mutations in genes like *Kras*, *Cdkn2a/b*, *Smad4* and *Trp53* and a large list of genes whose causal link to cancer is unknown. Interestingly, by screening a compound library we find that the identified oncogenic *Kras* mutation in Colon 26 cells correlates with its sensitivity to MEK inhibitors *in vitro* and *in vivo*. Our analysis of these mouse tumor exomes show that their manageable number of mutations could facilitate the identification of novel mutations or pathways driving tumor development. Furthermore, their use as tools is now enhanced as they can be used to create syngenic transplant models for utilization in drug discovery and validation. Finally, by showing that *Kras* mutant Colon 26 cells are sensitive to MEK inhibitors, we provide one proof-of-principle experiment that a platform containing targeted resequencing and drug screens could be a valuable addition in the clinic to devise anti-cancer drug schemes.

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INTRODUCTION

Studies in human cancers have identified several driver mutations that contribute to tumor development.¹ Interestingly, whole-genome sequencings of tumors have revealed that the most common recurrent mutations were already found before the next-generation sequencing was developed. On the other hand rare, but recurrent, mutations are starting to be identified, which ought to have a great impact on medicine. Moreover, targeted resequencing of either regions containing known cancer mutations or whole exomes, enables a much faster turnover. As recently shown, data generated from these analyses could impact clinical decision making in a near future.² In addition, new mutations in known or novel genes can also trigger development of new, targeted therapies.

The C57BL/6 mouse strain was the second mammal to have its genome sequenced.³ Next-generation sequencing therefore can be performed on targeted regions, but thus far, only few mouse tumor exome sequences have been reported.^{4,5} Because of the great utility of syngenic mouse transplant models for tumor biology and drug discovery studies, we set out to characterize three frequently used mouse tumor cell lines, which are transplantable in the strain they initially developed. Here, we report the completion of new mouse tumor exome sequences that show mutations involved in the tumorigenic process of these cells. We also provide proof-of-concept evidence that exome sequencing can identify mutations that predict responses to targeted therapies.

RESULTS AND DISCUSSION

Polymorphisms can affect exome sequencing data

Targeted resequencing holds promise as a method that could predict treatment regimens but thus far no patient has been treated based on results from exome sequencing.² Exome sequencing of inbred mouse tumor cell lines provide insight into the utility of these lines as tools for studies of gene function, to dissect signal transduction pathways and in validation of targeted therapies *in vitro* and *in vivo*. To gain preclinical proof-of-principle we sequenced three different mouse tumor cell lines: Panc02 pancreatic cancer cells, Colon 26 colon carcinoma and L1210 leukemia cells (Supplementary Table S1 for sequencing statistics, Supplementary Tables S2–S4 for mutations, respectively). All of these lines are transplantable in their respective syngenic strains C57BL/6 (Panc02), Balb/c (Colon 26) and DB/A (L1210). They have been used for decades in cancer research and in drug validation studies.

To develop one oncogenic mutation, it is likely that several others have to occur before or simultaneously. Other sources of mutations could be extensive cell culture under conditions that promote mutations. Our hypothesis was therefore that we would encounter thousands of mutations and that it would be difficult to identify driver mutations. Indeed, alignment of the Panc02 exome to the mm9 reference genome derived from the same strain, C57BL/6, revealed 1989 exonic single-nucleotide variations (SNVs), of which non-synonymous single-hits occurred in 605 genes

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(586 missense mutations, 19 stop gain; Supplementary Table S2). There were also 32 insertion or deletions (indels) resulting in frameshifts. However, the amount of SNV and indels is similar to the recently sequenced B16 melanoma cell line⁴ and comparable to many human tumors when polymorphism has been corrected for. Moreover, a high fraction of the observed mutations were transversions, indicative of mutations induced by the 3-methylcholanthrene used when Panc02 originally was developed.⁶

When analyzing the Colon 26 and L1210 sequences, we noted that the SNV was at least 20-fold more than in Panc02 upon alignment to the mm9 genome. To answer the question if this was due to polymorphism between the different strains, we built the Balb/c and the DBA/J genomes by replacing the mm9 SNVs and indels with the single-nucleotide polymorphisms of the respective strains downloaded from Sanger. Indeed, realignment of the Colon 26 exome data to the Balb/c-corrected reference genome reduced the amount of SNVs and indels and the final data is presented in Supplementary Tables S3 and S4.

Panc02 cells have genetic alterations in the transforming growth factor β pathway

As seen in Figure 1a and Supplementary Table S2, Panc02 exhibited a homozygous stop-gain mutation in *Smad4*, which is critical for mediating a growth suppressing transforming growth factor β or BMP signals. *SMAD4* mutations have previously been shown to occur in pancreatic and colon cancer but they are not actionable in predicting therapeutic responses. Besides *Smad4* no other classic mutation could be found but a copy number gain on chromosome 10 contained *Usp15* (Figure 1b and Supplementary Figure S1), which is known to amplify transforming growth factor β and mitogen-activated protein kinases (MAPK) signals.^{7,8} Moreover, from the COSMIC top-cancer gene list (http://www.sanger.ac.uk/perl/genetics/CGP/core_line_viewer?action=gene_list), Panc02 carried a heterozygous mutation in the oncogene *Gnas*, and a homozygous mutation in *Nkx2-1*, which was predicted to be damaging by the POLYPHEN software (<http://genetics.bwh.harvard.edu/pph2/>). However, mutation analysis of human cancer does not support a role of a mutation in this particular residue of *Gnas*. Furthermore, *Nkx2-1* mutations are more associated with lung cancer where Nkx2.1 can be both oncogenic and a metastasis suppressor. Therefore, the relevance of *Gnas* and *Nkx2-1* mutations require experimental validation before Panc02 cells can be considered tools for studies of these genes in cancer.

Another well-known cancer gene that was mutated was the oncogene *Braf* (heterozygous I313T). To address if this mutation rendered cells dependent on *Braf* signaling we screened a small drug library of 134 known inhibitors, including the *Raf* inhibitors PLX-4720 and PLX-4032 (Vemurafenib, Selleckchem, Houston, TX, USA). However, B-Raf inhibitors did not affect Panc02 cells, suggesting Panc02 cells are not dependent on B-Raf signaling (Supplementary Table S5). They did however, exhibit highly active MAPK signaling and sensitivity to some of the MEK inhibitors (Supplementary Table S5 and Figure 1c). As no receptor tyrosine kinase inhibitors had an effect on cell proliferation it is possible that the highly active MAPK pathway occurs independent of receptor tyrosine kinases. Rather, a candidate gene alteration driving MAPK signaling is the amplified *Usp15* gene (Figure 1b), as it is known to amplify both transforming growth factor β and MAPK signals.^{7,8}

L1210 cells have inactivating mutations of *Trp53* and *Cdkn2a/b*. The L1210 tumor was obtained in a female DBA/2 mouse after repeated paintings on the skin with 3-methyl-cholanthrene.⁹ It was initially serially transplanted in mice but later established in suspension culture. Our data confirms that our L1210 cells are of DBA origin, based on that many of the mutations

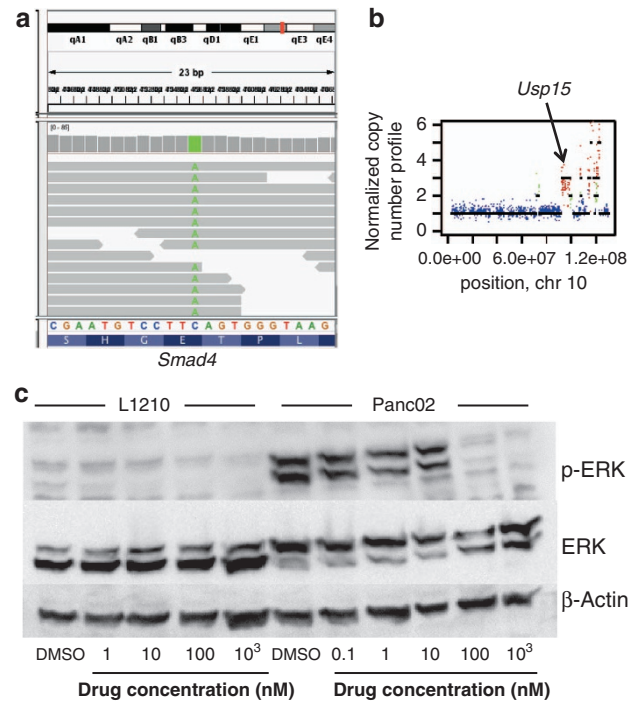


Figure 1. Panc02 cells have alterations in transforming growth factor β signaling. Panc02 (a kind gift from JW Greiner) cells were cultured in McCoy's medium supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, non-essential amino acids and 10 mmol/l HEPES and gentamycin. Genomic DNA was prepared from cells using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Next-generation sequencing was performed using the SureSelect Target Enrichment System Capture Kit (Agilent Technologies, Santa Clara, CA, USA) at BGI China on an Illumina HiSeq2000 (Illumina, Inc., Santa Clara, CA, USA). (a) Exome sequencing alignment data between the mm9 reference genome and Panc02 was visualized using the software IGV tools v2.0 (<http://www.broadinstitute.org/software/igv/home>). The homozygous *Smad4* mutation, resulting in a stop codon (TAA) was noted. (b) Schematic representation of copy-number variations of Chromosome 10, demonstrating an amplification of *Usp15*. (c) Western blot analysis of Panc02 and L1210 cells treated with GSK1120212 for 24 h. Antibody binding was visualized by enhanced chemiluminescence with the Luminata Forte reagent from Millipore (Merck Millipore, Billerica, MA, USA) and the LAS1000 imaging system (Fujifilm Life Science, FUJIFILM LAS and GE Healthcare Biosciences, are strategic alliance partners). Antibodies used were phospho-ERK and total ERK (both from Cell Signaling Technology, Inc., Danvers, MA, USA) and, as a loading control, β -Actin (Sigma, GE Healthcare Biosciences, Pittsburgh, PA, USA). L1210 leukemia cells (kind gift from O Heby) were cultured in RPMI-1640 medium with 10% FCS, 2 mmol/l L-glutamine and antibiotics.

found represented DBA single-nucleotide polymorphisms when comparing our exome sequences to the mm9 (C57BL/6) reference genome (Supplementary Table S4). Furthermore, FACS analysis shows that the cells express B220, confirming that it is a B-cell line (data not shown).

We found several mutations in known cancer genes but only the heterozygous mutations in *Trp53* (one inactivating frameshift mutation and one M240I mutation) encoding the p53 tumor suppressor and a large deletion of the *Cdkn2a/b* loci encoding p19Arf, p16Ink4a and p15Ink4b, are previously described (Supplementary Table S4 and Supplementary Figure S2). Moreover, many COSMIC top-cancer genes carried heterozygous mutations. Interesting genes previously implicated in hematological malignancies, included *Notch1*, *Wt1* and *Ezh2*. Mutations in these genes can be both inactivating and oncogenic depending on context.

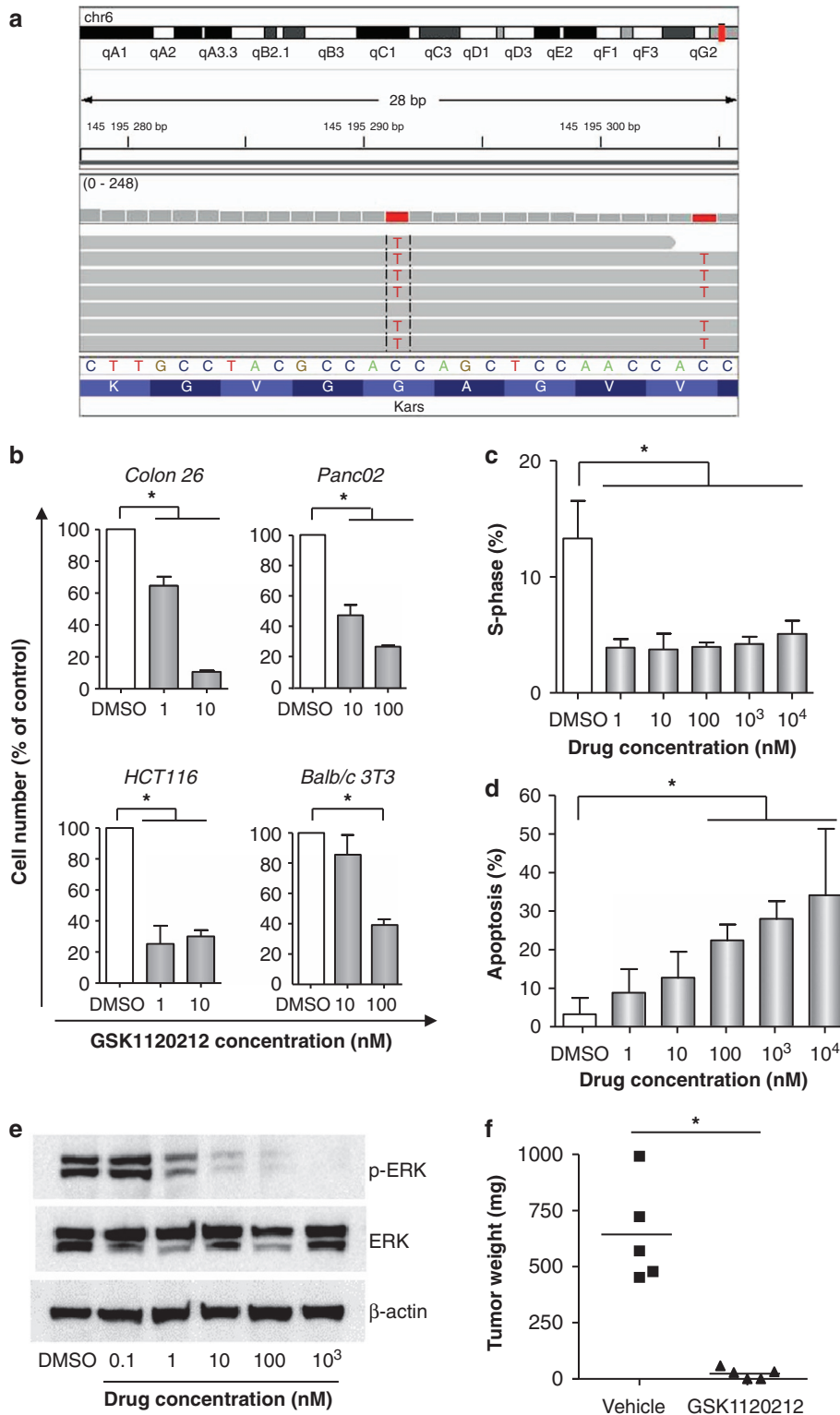


Figure 2. For caption see next page.

To see if the mutations acquired in L1210 cells sensitized them to targeted therapies, we subjected the cells to the focused drug library. Interestingly, compounds perturbing cell cycle checkpoints such as Polo-like kinase inhibitors, microtubuli-disrupting agents, Ksp inhibitors, Wee1 and Chk1 kinase were highly effective (Supplementary Table S5). Others, and we, have previously shown

that p53 deficiency, especially in the context of overexpression of the oncogene *Myc*, sensitizes cells to perturbations of the progression through the G2 and M-phase of the cell cycle. We therefore propose that p53 deficiency revealed by exome sequencing could predict sensitivity to targeted therapies directed against mitotic progression.

Figure 2. A *Kras* mutation predicts sensitivity to MEK inhibitors (a) Exome sequencing alignment data between the mm9 reference genome and Colon 26 was visualized using the software IGV tools v2.0. Note the heterozygous *Kras* mutation, resulting in an activating G12D substitution. (b) Colon 26 colon carcinoma (CLS Cell Lines Service GmbH, Eppelheim, Germany) were cultured in RPMI-1640 medium with 10% FCS, 2 mmol/l L-glutamine and antibiotics. Balb/c 3T3 fibroblasts were from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium with 10% FCS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and antibiotics. HCT116 (a kind gift from B Vogelstein) were cultured in McCoy's medium supplemented with 10% FCS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, non-essential amino acids and 10 mmol/l HEPES and gentamycin. Colon 26, Panc02, HCT-116 and Balb/c 3T3 cell lines were plated at individual densities (between 25 000–75 000 cells/cm²) and were allowed to attach before treatment with vehicle (DMSO) or the indicated concentration of GSK1120212 (Selleck Chemicals LLC). Forty-eight hours after start of treatment cells were counted by trypan blue exclusion. (c) Colon26 cells were treated for 16 h with indicated concentrations of GSK1120212. Cell nuclei were prepared with Vindelöv's solution, which also stained the cells with PI for analysis on a FACSCalibur flow cytometer. Shown are fractions of cells in S-phase determined in the FL2 channel. (d) Colon26 cells were treated for 40 h with indicated concentrations of GSK1120212. Cell nuclei were prepared, stained with PI and analyzed on a flow cytometer. Shown are fractions of cells exhibiting a sub-G1 DNA content. (e) Western blot analysis of Colon26 cells treated with GSK1120212 for 24 h using antibodies directed against phosphorylated and total ERK1/2. β -actin was used to confirm equal loading. (f) The *in vivo* experiments were performed in accordance with the Regional Animal Ethic Committee Approval 287/11 and 289/11. Balb/c mice were obtained from Harlan Laboratories (Rossdorf, Germany). To create a tumor model, 5 00 000 Colon 26 cells were mixed 1:1 with Matrigel and injected subcutaneously into 10 Balb/c mice. One week after injection tumors were visible (> 200 mm²) and treatment was initiated. GSK1120212 was dissolved in DMSO:PEG400:Chromophore:water, 1:10:10:29. Five mice were dosed with 1 mg/kg GSK1120212 and five mice were dosed with vehicle once daily for 4 days by intraperitoneal injections. Six hours after the last injection, tumors were excised and weighed. In all figures values statistically significant different from the control ($P < 0.01$, Student's *t*-test) are indicated with an asterisk (*).

Mutations identified by exome sequencing of Colon 26 cells can predict sensitivity to targeted therapies

The Colon 26 tumor developed as an undifferentiated carcinoma in a Balb/c female treated with nitrosomethylurethan.⁶ To investigate which genes and pathways are affected in these cells, we mined the top cancer gene list of the COSMIC database and compared it with our exome sequencing data. We found few mutations of known cancer genes, which could be explained by the dominance of the observed *Kras* G12D and loss of the *Cdkn2a* locus (Figure 2a and data not shown).

To gain insight if the *Kras* G12D would result in sensitivity to perturbations in known cell signaling pathways we performed a drug sensitivity screen. Interestingly, several MEK inhibitors induced cell death (Supplementary Table S5). To validate the drug screen, we treated the sensitive lines with GSK1120212, a third generation MEK inhibitor currently undergoing clinical trials against several solid tumors.¹⁰ Indeed, as seen in Figure 2b, Colon 26 were highly sensitive to GSK1120212. Furthermore, non-transformed Balb/c 3T3 cells and Panc02 cells were less sensitive than Colon 26, whereas HCT116, a human colon cancer cell line also carrying a *Kras* mutation, exhibited sensitivity. L1210 was not sensitive at all but did not have an activated MAPK-pathway (Figure 1c). Taken together, our data suggests that a Ras mutation sensitizes cells to MEK inhibitors. The sensitivity of Panc02 cells are also in accordance with a recent study,¹¹ showing that cells with an activate MAPK pathway are sensitive to MEK inhibitors.

To gain insight to the mode of action of GSK1120212 we analyzed Colon 26 cells by flow cytometry following treatment with GSK1120212. The earliest response was a cell cycle arrest at 16 h preceded by a change in morphology (Figure 2c and data not shown). On the second day of treatment the cells started to die by apoptosis (Figure 2d). Reassuringly, the same dose needed to inhibit ERK phosphorylation was sufficient to induce cell cycle arrest and apoptosis, suggesting that the effects were mechanism-based (Figure 2e). Moreover, a different third generation MEK inhibitor, TAK-733,¹² also suppressed ERK phosphorylation and induced cell cycle arrest followed by apoptosis (Supplementary Figure S3A–C). Taken together, we show that Colon 26 cells carrying a *Kras* mutation are sensitive to MEK inhibitors.

To investigate if the sensitivity to MEK inhibition also could manifest *in vivo*, we injected Colon 26 cells subcutaneously into the flank of 10 Balb/c females. One week after transplant, all mice had developed palpable subcutaneous tumors. Five of the mice received four daily injections with GSK1120212, whereas the rest received injections with vehicle. Already on the day of the forth injection, all treated tumors had regressed, and upon sacrifice,

remnants were visible in only three of the treated mice, whereas two showed no evidence of tumors (Figure 2f). To verify that cell death was mediated by apoptosis, we repeated the experiment and harvested tumors after 60 h (four injections) and performed immunohistochemistry on the remaining tumor. As seen in Supplementary Figure S4, a large amount of cells stained positive for cleaved caspase-3, demonstrating that they were undergoing apoptosis.

In this study, we have shown that exome sequencing can be useful in identifying how a tumor develops. We also demonstrate the powerful combination of sequencing data and functional testing by drug screening to identify and characterize actionable mutations. We propose that this platform could be made standardized for the clinic, which would facilitate the identification of the right treatment modality for the right patient. Importantly, as only a fraction of relevant mutations in cancer are therapeutically actionable, data from this platform could aid in the faster discovery of biomarker mutations that cannot be deduced from the current knowledge of cancer pathways. However, because of clonality and tumor heterogeneity, future studies will have to be performed to determine, which tumor types would benefit from such a platform.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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