Control for stochastic sampling variation and qualitative sequencing error in next generation sequencing analysis of KRAS actionable mutations

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Background: Clinical implementation of Next-Generation Sequencing (NGS) for analysis of actionable mutations in tumor tissues is challenged by poor control for stochastic sampling, library preparation biases and qualitative sequencing error. Potential sources of analytical variation due to stochastic sampling include low number of analyte copies loaded into library preparation and low number of library amplicons loaded into sequencer. Sequence error may be introduced by polymerase infidelity during amplification in library preparation and/or sequencing reaction. Inadequate control for these factors prevents establishment of reliable lower detection threshold for measurement of actionable mutations in cancer specimens. We recently developed a multiplex competitive PCR amplicon method for NGS library preparation that controls for these sources of analytical variation (Blomquist et al, 2013; Blomquist et al, 2015). Here we describe implementation of this method to determine lower limits for reliable measurement of KRAS codons 12 and 13 mutations in clinical samples.

Methods: DNA was extracted from FFPE samples containing KRAS mutation and serially diluted relative to FFPE-derived DNA without mutation. Each sample DNA was mixed with a known number of synthetic internal standard (IS) molecules for KRAS and multiple other targets, then multiplex PCR-amplified using primers spanning a 100 bp region for each target, including the KRAS region containing codons 12 and 13 (nucleotide positions 34-39). The PCR product for each sample was then loaded onto a Fluidigm AccessArray and each target, including KRAS, was amplified in a second round with single target primers. Each second round PCR product was loaded onto an Illumina HiSeq. Total KRAS molecule copies (i.e., wild-type and mutated) were measured in each sample relative to the known number of synthetic internal standard copies. Confidence limits for each value (percent mutated) were calculated based on an equation that controlled for number of copies loaded into library preparation and library amplicons loaded into sequencer. Technically-derived qualitative sequencing errors (i.e., base substitution, insertion and deletion) were measured at each codon 12/13 base position in both a) KRAS native template (NT) and b) synthetic IS. We previously determined that the frequency and type of sequence variation at each base position within each IS is concordant with that in respective NTs (r2 = 0.93). Results: For G>A mutations (e.g. 35-38G>A), the polymerase error frequency was about 0.1-0.3%. Thus, the lower limit for reliable detection of a two-fold difference would be above 0.5%. In contrast, for G>C mutations error frequency was <0.01% and as long as 50,000 amplifiable KRAS copies are loaded (approximately 500 ng of FFPE sample) into library preparation, and given sufficient sequencing space/sample ratio, it would be possible to reliably measure two-fold variation in fraction as low as 0.05% with 95% confidence.

Conclusion: The limit to lower detection of KRAS mutation fraction is determined by a) number of amplifiable mutated copies loaded into library preparation, b) number of library products loaded into sequencer, and c) ratio of
sequencing space/sample, and d) frequency of polymerase error at the nucleotide site bearing the mutation. In targeted NGS, synthetic competitive IS control for stochastic sampling at input of both target into library preparation and of target library product into sequencer, enable reduced sequencing space requirement, and control for qualitative errors generated during library preparation and sequencing. These controls enable accurate clinical diagnostic reporting of confidence limits and limit of detection for copy number measurement, and reduce sequencing space required for analysis.

Characteristics, oncogene dependency, and response to ponatinib of a novel transgenic mouse model of KIF5B-RET-induced lung adenocarcinoma

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Lung cancer is molecularly heterogeneous characterized by various genetic alterations. Many targetable mutations in lung adenocarcinoma occur at low frequencies, which limits timely recruitment of patients in clinical trials of precision therapy. Thus, preclinical animal models are invaluable for evaluation of novel targeted drugs. Recurrent RET fusions have been found in 1-2% of nonsmall cell lung cancer (NSCLC). Among them KIF5B-RET fusion is the most prevalent. To develop an animal model for evaluation of KIF5B-RET fusion in lung adenocarcinoma, we generated transgenic mice containing a doxycycline-inducible tetO-KIF5B-RET transgene. Induction of CCSP-rtTA/tetO-KIF5B-RET bitransgenic mice with doxycycline resulted in MRI- and CT-detectable lung adenocarcinoma in 4-5 months. KIF5B-RET-induced lung adenocarcinoma is characterized by desmoplastic reaction. Desmoplasia is associated with invasive lung adenocarcinoma and is found in human RET-fusion-positive lung adenocarcinoma, but it has not been observed in transgenic mouse models of EGFR1L858R, KrasG12D, or PTPN11E76K-induced lung adenocarcinoma. Using MRI, CT, and histological examinations, we evaluated oncogene dependence and the response to the multikinase RET inhibitor ponatinib in KIF5B-RET-induced lung tumors. Tumors regressed one month after Dox withdrawal or ponatinib treatment. Thus, we have established a transgenic mouse model of KIF5B-RET-induced lung adenocarcinoma. The KIF5B-RET-induced lung tumors are associated with desmoplasia, dependent on KIF5B-RET for maintaining the malignant phenotype, and respond to ponatinib treatment.

Mechanism of Notch and Sox2 in Kras driven lung adenocarcinoma in type II cells

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We have previously used cell lineage specific CreER mouse models to identify Type II cells as the cell-of-origin of KrasG12D induced lung adenocarcinoma. Using gain and loss of function genetic models, we discovered the active Notch signaling and low Sox2 dictate the ability of type II cells to proliferate and progress into adenocarcinoma upon KrasG12D activation. Here we provided further evidence that Notch signaling is required for KrasG12D induced Type II cells into a biopotent embryonic state. These Spc+Rage+ cells express specific lung embryonic development marker Sox9 and Ezh2. Additionally, genetical and chemical Sox2 upregulation profoundly inhibits KrasG12D-induced lung tumor formation in Type II cells. Sox2-expressing Type II cells are unable to dedifferentiating Type II cells when Kras is activated. In conclusion, our data suggest Kras can specifically transform Type II cells into lung adenocarcinoma by dedifferentiating Type II cells into Spc+Rage+ bipotent embryonic progenitor cells through activated Notch signaling. Unregulated Sox2 can alter Type II cells to proximal cell state, with subsequent loss of ability to dedifferentiate into these bipotent embryonic cells. Our findings could provide a new therapeutic strategy to target Kras activated lung cancer.

* Due to unforeseen circumstance, this poster was not presented.

ROR1 functions as a scaffold of caveolae and RTK-mediated survival signaling in lung cancer

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