Mutation detection in FFPE and plasma circulating DNA with a focused ALK-EGFR-KRAS Next-Generation Sequencing panel

Peng Fang, Agus Darwanto, Zhenyu Yan, Weihua Liu, Kimberly Pelak, Jessica Kristof, Philip C. Mack*, Sabita Sankar, Chad Galderisi and Jin Li
MolecularMD Corp., Cambridge, MA and Portland, OR; *UC Davis Cancer Center

Abstract A208

Introduction

Lung cancer is the most common cancer diagnosed and the leading cause of death from cancer worldwide. Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancers. Several contributing oncogenes including anaplastic lymphoma kinase (ALK), epidermal growth factor receptor (EGFR) and/or Kirsten rat sarcoma (KRAS) have been targeted by kinase inhibitors either approved or in development to treat NSCLC patients. Studies have shown that the types and locations of the mutations in ALK, EGFR or KRAS can serve as biomarkers to predict clinical response to these targeted therapies. Determination of mutation status for these oncogenes thus provides guidance for the choice of a potential therapy.

The poor quality of the DNA obtained from formalin fixed paraffin-embedded (FFPE) tumor tissue, and the limited quantity of the DNA obtained from the circulating plasma DNA, present technical obstacles to frequent and effective mutation profiling for the cancer patients. Next Generation DNA Sequencing (NGS) offers parallel sequencing of tens to hundreds of exons with sensitivities to at least 5%. The short read sequencing chemistry of NGS is ideal for FFPE DNA which is typically fragmented. In addition, the library preparation chemistry of the Ion AmpliSeq NGS platform, which requires only minimal DNA input, makes it possible for the circulating plasma DNA to be used in comprehensive mutation detection. Here we report the results from a proof-of-concept study using a custom Ion AmpliSeq NGS panel designed to sequence a range of clinically relevant exons in ALK, EGFR and KRAS genes in both FFPE DNA and plasma circulating DNA.

Materials and Methods

The cumulative 1.8 kb regions of interest (ROI) include exons 20-25 of ALK, exons 18-22 of EGFR, and exons 2-4 of KRAS, as well as the intronic 3 bp at intron-exon boundaries (**Table 1** and **Figure 1**). Two pools of the primers to sequence the ROI were designed using Ion AmpliSeq Designer. Two libraries were prepared for each DNA sample with Ion AmpliSeq Library Kit 2.0, using two separate pools of primers. The DNA input per library is 10 ng for cell line or FFPE DNA, or 0.5-2 ng for circulating plasma DNA. Each library was uniquely barcoded. The libraries were amplified on the OneTouch system using OneTouch OT2 200 Kit, enriched on the Ion One Touch ES, and sequenced on the Ion PGM using the Ion PGM Sequencing 200 Kit v2 and a 316 chip. The sequencing data were analyzed with Torrent Suite 3.4.2 and MMD proprietary analysis pipeline. The HapMap sample NA12878 was used as the negative control. The samples evaluated in this study include the Horizon Diagnostics (HZ-DX) Quantitative Multiplex DNA Reference Standard and ALK F1174L Genomic DNA Reference Standard, 8 FFPE DNA samples, and 4 plasma DNA samples (**Table 2-4**).

A series of experiments was conducted to evaluate the performance of the custom ALK-EGFR-KRAS NGS Panel, including the library coverage, the assay lower limit of detection (LOD) and accuracy, variant detection, and the concordance testing with commercially available assays including the Ion Torrent AmpliSeq Cancer Panel, the Illumina TrueSeq Amplicon Cancer Panel, and the Qiagen RGQ assay, as well as MolecularMD's proprietary droplet digital PCR (ddPCR) assay.

Figure 1. ROI targeted by the Custom ALK-EGFR-KRAS panel – Schematic of Amplicons by Primer Pools

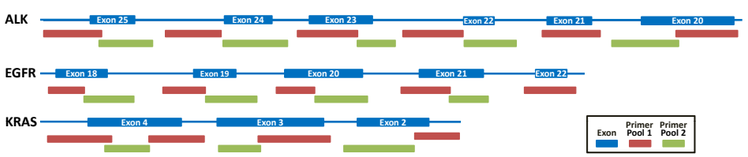


Table 1. ROI targeted by the Custom ALK-EGFR-KRAS panel

Gene	Exons	Amino Acid Residue Covered
ALK	20	1058-1120
ALK	21	1120-1150
ALK	22	1151-1172
ALK	23	1172-1215
ALK	24	1216-1248
ALK	25	1248-1279
EGFR	18	688-728
EGFR	19	729-761
EGFR	20	762-823
EGFR	21	824-875
EGFR	22	876-901
KRAS	2	1-37
KRAS	3	38-97
KRAS	4	97-150

Table 2. Cell line DNA samples

Cell Line Name	Source	Genes With Mutation	Mutations in Protein (COSMIC)
NA12878	Coriell	N/A	N/A
Quantitative Multiplex DNA Reference Standard- Beta version, September 2012	Horizon Diagnostics	EGFR	G719S
		EGFR	T790M
		EGFR	L858R
		EGFR	Exon 19 del
		KRAS	G13D
		KRAS	G12D
ALK F1174L Genomic DNA Reference Standard	Horizon Diagnostics	ALK	F1174L

Table 3. FFPE DNA samples

ID	Tissue	% Tumor	Source
MMD-2-4-021	Colon	90	Bioserve
MMD-2-2-033	Lung	90	Bioserve
MMD-2-2-031	Lung	90	Bioserve
MMD-2-2-030	Lung	95	Bioserve
MMD-2-4-025	Colon	90	Bioserve
MMD-2-2-028	Lung	90	Bioserve
MMD-2-2-027	Lung	85	Bioserve
MMD-2-4-020	Colon	N/A	Bioserve

Table 4. Circulating plasma DNA samples

ID	Patient	Source
CT013601	Lung Cancer	UC-Davis
CT013682	Lung Cancer	UC-Davis
CT09980	Lung Cancer	UC-Davis
CT13946	Lung Cancer	UC-Davis

Results

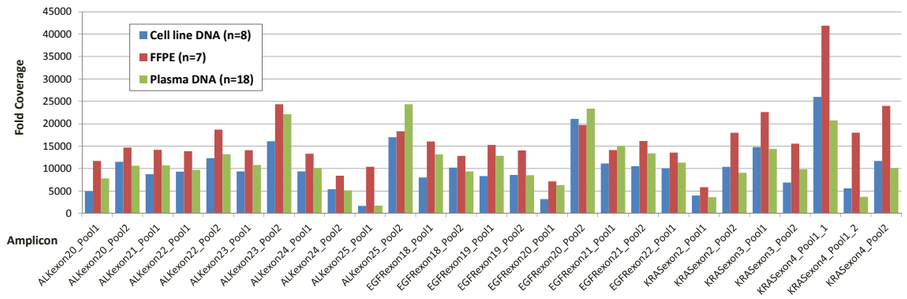
ROI and Library Coverage

In the four sequencing runs performed with 316 chip in this study, the library coverage ranges from ~5000x read depth to ~15000x read depth (**Table 5**). The coverage across the ALK-EGFR-KRAS NGS Panel ROI was uniform, and above 98.8% amplicons had more than 1000x coverage, in the libraries prepared from the cell line DNA, the FFPE DNA, and the circulating plasma DNA tested, and with both Primer Pools (**Table 5** and **Figure 2**).

Table 5. ROI coverage in the sequencing runs performed

Run	# Samples on Chip	Chip Type	Mean Library Coverage	Average ALK Coverage	Average EGFR Coverage	Average KRAS Coverage	% Amplicons with >1000x Coverage	DNA Type
MMD-109	6	316	10780 ± 3203	11989	13165	9595	98.8%	Plasma
MMD-115	7	316	15014 ± 4546	14503	14143	20749	100.0%	FFPE
MMD-118	10	316	9489 ± 2539	9664	11013	10146	98.9%	Plasma, Cell lines
MMD-119	10	316	9189 ± 2009	11490	11857	11528	98.9%	Plasma, Cell lines

Figure 2. Average coverage across amplicons



Assay LOD and Accuracy

The Quantitative Multiplex DNA Reference Standard from Horizon Diagnostics (Beta version), contains 6 mutations in EGFR and KRAS, with the mutation frequencies quantified by ddPCR. The ALK-EGFR-KRAS NGS Panel was able to accurately identify all of the expected mutations at approximately the expected frequencies, with frequencies as low as 1% (**Table 6**). In addition, another Genomic DNA Reference Standard from Horizon Diagnostics, the ALK F1174L with 50% allele frequency, was serially diluted into NA12878 to generate samples with expected frequencies of 10%, 3% and 1%. The DNA samples of these dilutions were sequenced with ALK-EGFR-KRAS NGS Panel, and the results indicated that the ALK variant was observed with approximately the expected frequency (**Table 7**). Additionally, a variant in KRAS, which was in the HCT-116 cell line harboring the engineered ALK F1174L mutation, was also detected at the expected frequency (**Table 7**).

Table 6. Variants detected in Quantitative Multiplex DNA Reference Standard

Gene	Amino Acid Change	Expected Variant Freq (%)	ALK-EGFR-KRAS NGS Panel Variant Freq (%)	NGS Panel Coverage
EGFR	G719S	24.5	23.4	4471
EGFR	T790M	1	0.7	6663
EGFR	L858R	2.5	1.8	3954
KRAS	G13D	16	15.4	3672
KRAS	G12D	5	6.3	3740
EGFR	E746-A750del (Exon 19)	2	1.2	1996

Table 7. Variants detected in ALK F1174L Genomic DNA Reference Standard

Gene	Amino Acid Change	Expected Variant Freq (%)	ALK-EGFR-KRAS NGS Panel Variant Freq (%)	NGS Panel Coverage
ALK	F1174L	10	9.7 ± 1.8	16835 ± 1334
KRAS	G13D	10	9.9 ± 1.1	9810 ± 2570
ALK	F1174L	3	4.8 ± 0.7	15648 ± 42
KRAS	G13D	3	3.7 ± 0.1	10368 ± 2452
ALK	F1174L	1	1.3 ± 0.5	14198 ± 1650
KRAS	G13D	1	1.1 ± 0.2	9448 ± 3888

Variant Detection in FFPE DNA Samples

Eight FFPE DNA samples were blinded and sequenced with the ALK-EGFR-KRAS NGS Panel (10 ng input per primer pool). Five unique KRAS mutations were detected in these samples, which were confirmed by the results from the commercial cancer panels (**Table 8**). In addition, the frequencies observed for these mutations were comparable to the frequencies observed for these mutations by either of the cancer panels. No additional mutations were identified in these samples by the ALK-EGFR-KRAS NGS Panel, which is consistent with the results from the commercial cancer panels, although the commercial panels only partially cover the ROI covered by the ALK-EGFR-KRAS NGS Panel.

Table 8. Variant detected in FFPE DNA and concordance

Sample Name	Gene	Amino Acid Change	Ion Cancer Panel Variant Freq (%)	Illumina Cancer Panel Variant Freq (%)	ALK-EGFR-KRAS NGS Panel Variant Freq (%)	NGS Panel Coverage
MMD-2-4-021	KRAS	Q61H	32.5	29.67	30.7	17325
MMD-2-2-033	KRAS	G12D	35.6	37.22	33.5	23554
MMD-2-2-031	KRAS	G12C	49.6	45.94	47.4	24302
MMD-2-2-030	KRAS	G12V	26.5	25.08	26.9	21920
MMD-2-4-025	KRAS	G12D	57.0	57.03	54.5	20901
MMD-2-2-028	KRAS	G12C	26.0	25.2	31.5	5839
MMD-2-2-027	KRAS	G12C	25.0	25.08	24.2	10148
MMD-2-4-020	KRAS	G13D	24.2	22.93	24.1	13719

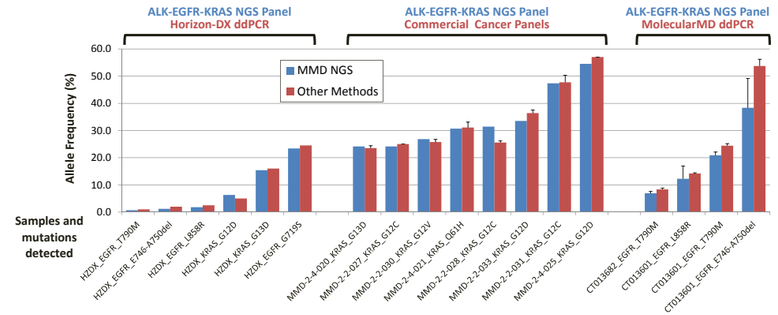
Variant detection in Circulating Plasma DNA

A total of six plasma DNA samples, collected from four NSCLC patients, were sequenced with the ALK-EGFR-KRAS NGS Panel (1-2 ng DNA input per primer pool). Several EGFR mutations, including T790M, L858R, and the exon 19 E746-A750 deletion, were detected by the ALK-EGFR-KRAS NGS Panel in five plasma DNA samples (**Table 9**). Each of these variants was confirmed by the Qiagen RGQ assay and MolecularMD's proprietary EGFR ddPCR assay. A single EGFR exon 19 deletion identified by the Qiagen RGQ assay was not seen in either the ALK-EGFR-KRAS NGS Panel or the ddPCR assay. No other potential false negatives or false positives were identified in the custom ALK-EGFR-KRAS NGS Panel.

Table 9. Variant detection in circulating plasma DNA and concordance

Sample Name	Gene	Amino Acid Change	ALK-EGFR-KRAS NGS Panel Variant Freq (%)	NGS Panel Coverage	RGQ call	ddPCR Variant Freq (%)
CT013601	EGFR	E746_A750del	38.3 ± 10.8	1979 ± 14	E746_A750del	53.8 ± 2.5
	EGFR	T790M	20.9 ± 1.2	22622 ± 11314	T790M	24.5 ± 0.8
CT013682	EGFR	T790M	6.9 ± 0.7	24007 ± 2261	T790M	8.4 ± 0.5
	EGFR	L858R	12.3 ± 4.7	15707 ± 5538	L858R	14.2 ± 0.3
CT09980	EGFR	None	None	None	E746_A750del	None
CT13946_8	None	None	None	None	None	None
CT13946_9	None	None	None	None	None	None
CT13946_10	None	None	None	None	None	None

Figure 3. Concordance between the NGS panel and the other methods



Concordance with Other Methods

In this feasibility study, both the mutations and their frequencies called by the ALK-EGFR-KRAS NGS Panel in the DNA samples tested, including the Horizon Diagnostics reference DNA samples, the FFPE DNA samples, and the circulating plasma DNA samples, were concordant with those from the other methods, including the Horizon Diagnostics ddPCR (for the Horizon Diagnostics reference DNA samples), the Ion Torrent and Illumina commercial cancer panels (for the FFPE DNA samples), and the Qiagen RGQ assay and MolecularMD proprietary EGFR ddPCR assay (for the circulating plasma DNA) (**Figure 3**). Only one additional mutation (the EGFR exon 19 deletion) identified by RGQ was not detected by the ddPCR assay or the NGS Panel (**Table 9**). No potential false positives were identified in this study.

Conclusions

- This study demonstrates the feasibility of creating a sensitive and specific ALK-EGFR-KRAS focused NGS assay that covers broader regions of the target genes than the hotspots represented in the commercial panels.
- With a minimum total DNA input of 20 ng of FFPE DNA, or 1 ng of the plasma circulating DNA, the assay was able to detect both SBS and small indels in the targeted regions, with the LOD of ~1%.
- Given the low DNA input requirements and the capability of plasma-based testing, this assay and other custom NGS panels may enable routine monitoring of mutation status for relevant genes in patients with various solid tumors, and may ultimately inform clinical decision-making.

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