RNA expression based screening of ALK Gene Fusion from NSCLC FFPE samples

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Abstract

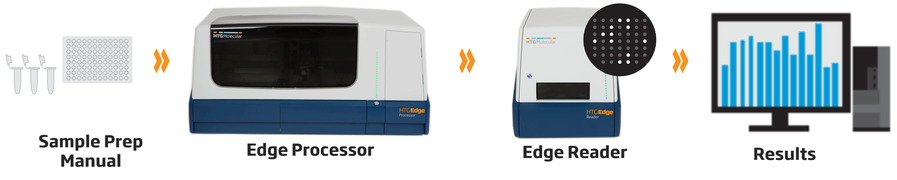
The ALK gene fusion results in the constitutive activation of the oncogenic ALK kinase activity through the expression of 3`ALK gene downstream of fusion partners like EML4 and KIF5B. Due to this rearrangement, fusion mRNA show high expression of 3` regions compared to 5` region of ALK gene and can be used as an indicator for presence of gene fusion. However, due to the low intrinsic expression of normal ALK mRNA, detection of the 5′ region of the ALK mRNA requires a highly sensitive assay system. To achieve this high sensitivity, we adapted the quantitative nuclease protection assay to combine biotinylated tiled probes for 3′ and 5′ ALK mRNA with the use of streptavidin conjugated polymeric horseradish peroxidase for detection.

This assay can robustly detect baseline levels of ALK mRNA from normal and ALK-negative NSCLC specimen. We then tested the ability of the assay to measure the over expression of 3′ ALK gene by spiking in 3′ and 5′ *in vitro* transcribed (IVT) ALK RNAs into normal lung samples. Signal over baseline was detected for ALK5` and ALK3` IVT at attomolar concentrations even in the presence of a ratio of 95:5 in the background of normal lung tissue. Known ALK fusion cell lines also showed exquisite sensitivity for detection of fusion by comparing 5′ and 3′ expression ratios with sample input as few as 65 cells per reaction.

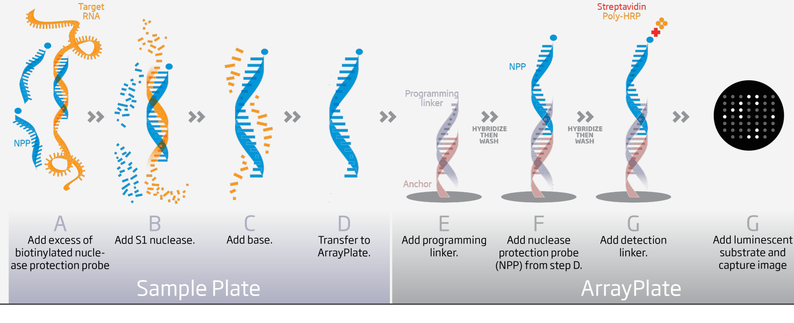
We screened 293 (222 Adenocarcinoma, 47 Squamous cell carcinoma and 24 other NSCLC subtypes) surgically resected NSCLC FFPE clinical samples from multiple sources for presence of EML4-ALK fusions. Endogenous ALK mRNA expression was detectable in all samples with 12 (4.09 %) samples showing ALK fusion based on aberrant overexpression of 3′ region compared with 5′ region. All of the 12 samples were of the adenocarcinoma or large cell subtype consistent with reported ALK fusion epidemiology (table). Confirmation studies with positives show greater sensitivity of detection of ALK fusion than is possible currently by FISH.

HTG Edge System & Chemistry

Automation



Chemistry



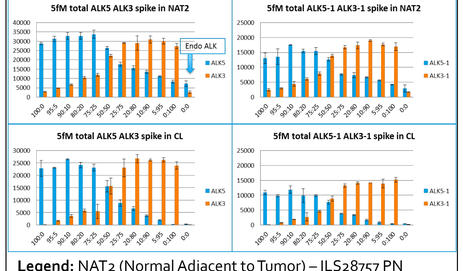
ALK Gene Fusion Assay

Methodology:

- ALK translocations (EML4-ALK; KIF5B-ALK) in NSCLC result in high expression of 3` mRNA region versus 5` mRNA region of ALK. This can be used as an indicator for presence of gene fusion.
- We performed quantitative nuclease protection assay using tiled biotinylated probes for 3` and 5` ALK mRNA
- ALK 5` probes were tiled across exon 1 and ALK 3` probes were tiled across exons 20, 25 and 29 of ALK mRNA (NM_004304)
- After nuclease protection, remaining probes were detected with streptavidin conjugated polymeric horseradish peroxidase
- To increase confidence and accuracy 2 microarray spots were tiled with non-identical probes for each of the ALK5` and ALK3` regions (ALK5` and ALK5`-1; ALK3` and ALK3`-1).

Performance testing :

- Assay performance (ability to measure the over expression of 3` ALK) was tested by spiking *in vitro* transcribed (IVT) ALK RNAs into normal lung FFPE samples and chicken liver FFPE lysate.
- Individual ALK 5` and ALK3` IVT RNAs were spiked into normal adjacent tumor specimen and Chicken Liver FFPE lysates (as surrogate matrix) at different ratio concentrations.



Legend: NAT2 (Normal Adjacent to Tumor) – ILS28757 PN
CL – Chicken Liver FFPE lysate

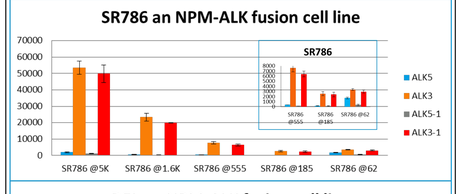
Conclusions

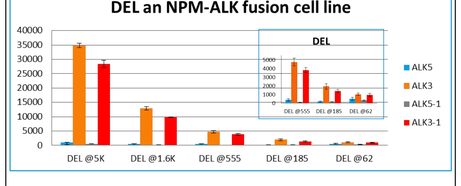
- High assay sensitivity allowed detection of low endogenous ALK mRNA expression robustly in normal lung and ALK negative NSCLC specimens.
- Signal over the baseline was detected for ALK5` and ALK3` IVT at attomolar concentrations even in the presence of 95:5 in the background of normal lung tissue.
- Data from these IVT spike-in experiments show good sensitivity and specificity ALK5 and ALK3 detection.

Detection of ALK fusion in cell line samples

- We procured NPM-ALK fusion expressing lymphoma cell lines from Leibniz Institute of DSMZ – German Collection of Microorganisms and Cell cultures.
- Cultured cells were lysed in HTG lysis buffer at 4million cells per mL.
- Cell lines were tested for ALK 3` and ALK5` expression at multiple concentrations (5000, 1666, 555, 185 and 62 cells per reaction).

Cell Line Testing:





Conclusions

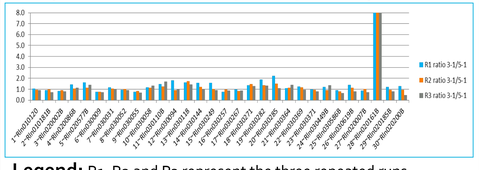
- SR-786 and DEL lymphoma cell lines were known to harbor NPM-ALK gene fusion.
- NPM-ALK gene fusions (Nucleophosmin – Anaplastic lymphoma Kinase) are associated with 40% cases of the Anaplastic Large-Cell Lymphoma (ALCL) patients.
- Both SR-786 and DEL cell lines showed exquisite sensitivity for detection of ALK fusion with as low as 65 cells per reaction.
- Cell line data demonstrate that our methodology could be used for detection of all ALK gene fusion variations including novel unknown ALK gene fusions.

Detection of ALK fusion in NSCLC FFPEs

NSCLC FFPE sample ALK fusion screen:

- 293 NSCLC FFPE samples from multiple sources were screened.
- Multiple NSCLC subtypes were included in the study
  - 222 adenocarcinoma
  - 47 Squamous cell carcinoma
  - 24 other NSCLC and non-NSCLC subtypes.
- Samples represent both Caucasian and Asian population.
- FFPE samples from 5mm sections were macro-dissected for tumor enrichment.
- Samples were tested at 0.15cm² of tissue per reaction.

Sample NSCLC FFPE ALK3`/ALK5` Ratio plot:



Legend: R1, R2 and R3 represent the three repeated runs.
- Sample 020161 shows presence of ALK fusion.
- Data shows assay has good reproducibility.

Results:

- ALK fusion screen showed - 279 negative, 12 positive and 2 borderline.
- All positive and borderline samples were - Adeno (or) Large Cell.
- Patient's age ranged from 27 - 71

ALK fusion positive samples epidemiology:

Sample ID	NSCLC Subtype	P63 IHC	TTF-1 IHC	Race	Sex	Histology	FISH ALK Translocation
HTG2031	ADENO	-	+	Caucasian	Female	NOS	Negative
HTG2065	ADENO	+	+	Caucasian	Male	Poorly differentiated	Failed
HTG1938	ADENO	+	+	Asian	Female	Undifferentiated	**Positive**
HTG1873	ADENO	+	+	Caucasian	NA	Adenosquamous	Negative
HTG1882	ADENO	+	+	Caucasian	Male	Poorly differentiated	Negative
HTG1874	ADENO	-	+	Caucasian	Male	Adenosquamous	Negative
HTG1880	ADENO	+	+	Caucasian	Female	NOS	Negative
HTG1888	ADENO	+	+	Caucasian	Female	NOS	**Positive**
HTG1822	ADENO	NA	NA	Asian	Male	BAC	Failed
HTG1970	ADENO	NA	NA	Asian	Male	BAC	Failed
HTG1760	Large Cell	NA	NA	Asian	Female	Undifferentiated	Failed
HTG1649	Large Cell	-	-	Asian	Male	Poorly differentiated	Failed

Conclusions

- We report a high sensitive ALK gene fusion screening assay, that could be extended for Fine Needle Aspirate (FNA) samples.
- Confirmation studies with positives show greater sensitivity of detection of ALK fusion than is possible currently by FISH.
- This screening assay could be used for detection of all ALK gene fusion variations including novel or unknown ALK gene fusions.
- ALK fusion array could be expanded to detect other clinically important gene fusions in NSCLC such as of RET, ROS1 and NTRK1.