Introduction

Circulating tumour DNA (ctDNA) is rapidly becoming established as an invaluable tool to supplement conventional biopsies for molecular characterization and monitoring of solid cancers, particularly for cancers such as non-small cell lung cancer (NSCLC), where tumour tissue is limited or unobtainable. As ctDNA may be a small fraction of total cell-free DNA (cfDNA), sensitive methods are required for its reliable detection. To overcome these challenges, we have developed an enhanced tag amplification panel sequencing method (enhanced TAm-Seq™). Here we present analytical validation of this technology across two clinical laboratories based in the UK and US to support its use in clinical applications.

What is enhanced TAm-Seq?

Enhanced TAm-Seq (eTAm-Seq™) combines efficient library preparation and statistical-based analysis algorithms to identify and quantify cancer mutations. Analysis was performed using a 35-gene panel including cancer hotspots, entire coding exons and copy number variants (Fig 2).

Analytical performance and validation of an enhanced TAm-Seq™ circulating tumour DNA sequencing assay across two laboratories

Assay validation

We have performed analytical validation of eTAm-Seq in two specific sensitivity, limit of detection (LoD), and inter-operator variability for detection of SNVs and indels. Analysis was performed using plasma DNA from healthy controls and Horizon TruQ-2 reference cell-free DNA carrying mutations in cancer-related genes as known allele fractions (AfS). eTAm-Seq was performed across two laboratories by five different operators on different days and sequenced on different NGS runs. One operator performed eTAm-Seq at both laboratories.

cDNA quantification of NSCLC patient plasma

The rationale for determining DNA input amounts used in validation experiments was based on dPCR quantification of cfDNA levels routinely obtained from NSCLC patients in a 10 ml EDTA blood draw. DNA yields ranged from 1,000–290,350 Amplifiable Copies/10 mL blood. (Fig 3). Based on this, three different input amounts were used—Low (2,000 AC—95% samples), Medium (8,000 AC—45% samples) and High (16,000 AC—45% samples).

Analytical validation performance

To assess sensitivity, Horizon Tru-Q 7 (Low input, undiluted AF majority at 25−2%) and Tru-Q 2 samples (Medium, undiluted AF majority at 32−3%) were diluted to AF 0.25−3.25%. These were then <200ng to mimic fragmented cDNA. Dilutions were prepared using Horizon Tru-Q 7 wild-type DNA as diluent. Fig 4 shows that AFs from eTAm-Seq have high sensitivity, with 95% (95% CI 99.0±100%) detected in low input samples; 100% (95% CI 97.5±99.5%) detected in Medium, and 95.4% mutations (95% CI 91.0±97.8%) detected in High input samples from the UK validation (top). Comparable results from the US lab are shown below, with 99.7% (95% CI 98.6±99.8%) detected in low input samples; 100% mutations (95% CI 98.7±100%) detected in Medium, and 97.2% mutations (95% CI 91.3±94.7%) detected in High input samples.

Analytical performance and validation of eTAm-Seq across 2 clinical labs.

Dilutions were prepared (Medium, undiluted, AF majority at 25−2.5% & High, undiluted, AF majority at 25−2%) and Tru-Q 2 samples (Medium, undiluted AF majority at 32−3%) were diluted to AF 0.25−3.25%. These were then >200ng to mimic fragmented cDNA. Dilutions were prepared using Horizon Tru-Q 7 wild-type DNA as diluent. Fig 4 shows that AFs from eTAm-Seq have high sensitivity, with 95% (95% CI 99.0–100%) detected in low input samples; 100% (95% CI 97.5–99.5%) detected in Medium, and 95.4% mutations (95% CI 91.0–97.8%) detected in High input samples from the UK validation (top). Comparable results from the US lab are shown below, with 99.7% (95% CI 98.6–99.8%) detected in low input samples; 100% mutations (95% CI 98.7–100%) detected in Medium, and 97.2% mutations (95% CI 91.3–94.7%) detected in High input samples.

Analytical validation performance

In analysis of undiluted samples, eTAm-Seq AFs were compared to AFs based on Horizon dPCR data, and show good correlation (Fig 6).

Clinical experience using eTAm-Seq

Fig 7 shows the allele fraction and spectrum of mutations identified in 153 patients with Stage II IV NSCLC, highlighting that 49.2% of mutations identified by eTAm-Seq in this patient population had AF >1%, with 36.4% having AF >0.5%. These mutations would routinely be missed using less sensitive assays. A broad spectrum of mutations across 24 different genes, including common EGFR (20.1%), uncommon EGFR resistance mutations (77900 (13.7%), C797S (3.3%), were identified. This combined patient dataset is derived from prospectus studies of NSCLC patients in first and second-line treatment settings, including an EGFR-positive cohort (n=55) being monitored for treatment response. Resistance mutations were tested using cfDNA (Remon J et al). Liquid biopsy for molecular profiling of mutations in non-small cell lung cancer (NSCLC) patients lacking tissue samples, AACR 2016, New Orleans, US, April 2016.

Summary

- cfDNA has potential as a liquid biopsy to supplement conventional tissue biopsies for non-invasive molecular stratification, monitoring response to treatment and detection of resistance mutations, and has particular utility in patients where tissue is limited.
- Enhanced TAm-Seq is able to detect and quantify cancer mutations in plasma. Analytical validation studies presented here show that eTAm-Seq is a highly sensitive method, with identification of ~97.6% point mutations at ~0.5% AF across both laboratories and limit of detection (LoD) of 0.25%.
- Analytical validation across 2 clinical laboratories in the UK & US showed good concordance, demonstrating the robustness of the eTAm-Seq assay.
- Analysis of 153 Stage II IV NSCLC patients show that eTAm-Seq is able to detect mutations at low allele fractions that would be routinely missed using less sensitive assays.
- These data demonstrate the analytical validity of the enhanced TAm-Seq assay and support its use in clinical applications.