The use of improved and complete enrichment co-amplification at lower denaturation temperature (ICE COLD-PCR) method for the detection of EGFR and KRAS mutations from cell-free plasma DNA of non-small cell lung cancer (NSCLC) patients

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Background: Identification of specific molecular alterations from cell free plasma DNA (cfpDNA) holds tremendous potential as a noninvasive method to assess tumor genotype. We evaluated whether ICE COLD-PCR (ICP) can be used to identify EGFR and KRAS mutations from cfpDNA in patients enrolled in the BATTLE research clinical trial. Methods: Tissue genotyping of KRAS (Exons 2, 3) and EGFR (Exons 18 - 21) on DNA extracted from formalin-embedded tumor tissue was determined using PCR-based sequencing analysis, with lower limit of sensitivity of detection of 20%. Genotyping of cfpDNA was determined using ICP for mutation enrichment followed by Sanger sequencing for mutation detection, with limit of detection of 0.01%.

Results: DNA was isolated and extracted from 154 available plasma samples with matched tumor genotype; with volumes ranging 0.2–0.7 mL. For the overall population with or without mutations, a concordance of 92%, 95%, and 86% was observed with tissue genotypes for EGFR Exon 19, EGFR Exon 21 and KRAS Exon 2, respectively. Mutation specific sensitivities were 80% for EGFR-19del, 44.4% for EGFR-21-L858R, and 34.4% for KRAS-2. Conclusions: The use of cfpDNA for the determination of important EGFR and KRAS mutations provides a non-invasive method which may assist physicians with clinical care for cancer patients. The results from this analysis are encouraging, but, regardless of the methodology used for mutation detection in cfpDNA, additional assay standardization is required for clinical use:

- Amount of extracted DNA used per assay.
- Determination of initial plasma volume for extraction.
- Ability to multiplex amplicons of interest.
- The use of improved and complete enrichment co-amplification at lower denaturation temperature (ICE COLD-PCR) method for the detection of EGFR and KRAS mutations from cell-free plasma DNA of non-small cell lung cancer (NSCLC) patients.
- For this study, the failure in obtaining results may be due to:
  - Limited DNA in sample due to starting extraction volume.
  - PCR product was observed; however, poor sequencing results were obtained (i.e. baseline issues).
  - Efficiency of the specific ICP reaction. For this study, EGFR Exon 21 had the highest failure rate and has subsequently been redesigned without a loss in sensitivity.


Methods

ICE COLD-PCR Sequencing

Step 1: All DNA is denatured to single strands.
Step 2: The RS-oligo binds to one strand of the wild-type and mutant sequences: mutant:RS-oligo forms a heteroduplex.
Step 3: The reaction is incubated at the Tc: the mutant:RS-oligo denatures but the wild-type:RS-oligo remains bound.
Step 4: The forward and reverse PCR primers will bind to both strands of the mutant DNA, but only one strand of the wild-type DNA.
Step 5: Extension of the PCR primers along the mutant and wild-type DNA sequences. The mutant sequence will undergo exponential amplification while the amplification of the wild-type DNA sequences.

Results

Summary of Mutation Analysis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>EGFR Exon 19 (del)</th>
<th>EGFR Exon 21 (L858R)</th>
<th>KRAS Exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Mutation Detected</td>
<td>135</td>
<td>98</td>
<td>115</td>
</tr>
<tr>
<td>Mutation Matched</td>
<td>8/10</td>
<td>4/9</td>
<td>11/32</td>
</tr>
<tr>
<td>Concordance</td>
<td>92%</td>
<td>95%</td>
<td>86%</td>
</tr>
</tbody>
</table>

Sensitivities

- Mutation in Tissue Only: 80.0% 44.4% 34.4%
- Mutation in Plasma Only: 2 5 21
- Various deletions L858R(2) G12C(4), G12D(1), G12A(1), G13D(1)
- ICP failure 1* 23* 9*

Summary & Clinical Significance

- The use of cfpDNA for the determination of important EGFR and KRAS mutations provides a non-invasive method which may assist physicians with clinical care for cancer patients.
- The results from this analysis are encouraging, but, regardless of the methodology used for mutation detection in cfpDNA, additional assay standardization is required for clinical use:
  - Determination of initial plasma volume for extraction.
  - Amount of extracted DNA used per assay.
  - The influence of tissue heterogeneity on mutation detection in cfpDNA.
- Ability to multiplex amplicons of interest.
- These results demonstrate the feasibility in the use of ICP for mutation determination from cfpDNA of NSCLC patients. Validations of these updated assays are going.
- EGFR T790M - Exon 20 verification and validation are currently on-going.