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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Abstract

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 form paraffin-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 such as initial plasma volume for extraction, amount of extracted DNA and the influence of tissue heterogeneity versus cfpDNA in mutation detection are needed prior to routine clinical use. Overall, these results demonstrate the feasibility in the use of ICP for mutation determination from cfpDNA of NSCLC patients. Clinical trial information: NCT00409968, NCT00411671, NCT00411632, NCT00410059, and NCT00410189.

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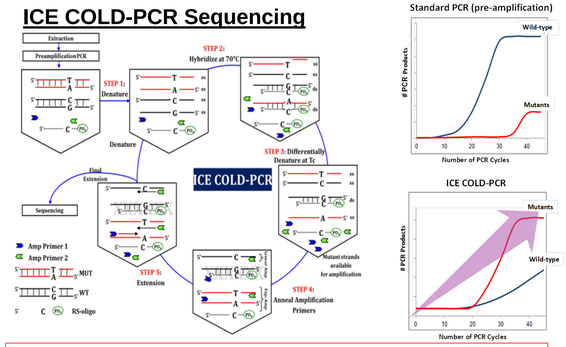
Methods

All patients provided signed, written informed consent for this laboratory-based research study approved by UTMDACC IRB under BATTLE clinical trial protocols.

- cfpDNA mutation analysis was done under blinded fashion
- 154 available plasma samples with matched tumor genotype
- Plasma volume ranged from 200 – 700 μL
- DNA extraction: QIAamp circulating nucleic acid kit (Qiagen)
- ICP methods for *EGFR* Exons 19, 21 and *KRAS* Exon 2 are used for this study

UTMDACC EGFR Mutation Tissue Pathology Analysis: PCR-based EGFR exon 18 to 21 and KRAS DNA sequencing analysis was performed on DNA extracted from paraffin-embedded tumor tissue blocks. The lower limit of sensitivity of detection is approximately one mutated cell per five total cells in sample (20%).

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: Anneal the PCR primers. The forward and reverse PCR primers will bind to both strands of the mutant DNA, but only one strand of the wild-type.
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 sequence will be linear.
Step 6: Perform standard Sanger Sequencing reactions.
Step 7: Analyse using a DNA sequencer.

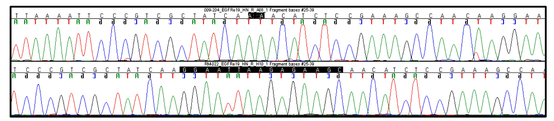
Results

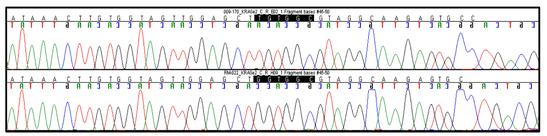
Summary of Mutation Analysis

	EGFR Exon 19 (del	EGFR Exon 21 (L858R)	KRAS Exon 2
No Mutation Detected Matched	135	98	115
Mutation Matched	8/10	4/9	11/32
Concordance	92%	95%	86%
Sensitivities	80.0%	44.4%	34.4%
Mutation in Tissue Only	2	5	21
Mutation in Plasma Only	7	2	7
	Various deletions	L858R(2)	G12C(4), G12D(1), G12A(1), G13R(1)
ICP failure	1*	23*	9*

- 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.

References:
Kim ES, Herbst RS, Wistuba II, Lee JJ, Blumenschein Jr GR, Tsao A, Stewart DJ, Hicks ME, Erasmus J Jr, Gupta S, Alden CM, Liu S, Tang X, Khuri FR, **Tran HT**, Johnson BE, Heymach JV, Mao L, Fossella F, Kies MS, Papadimitrakopoulou V, Davis SE, Lippman SM, Hong WK. The BATTLE Trial: Personalizing Therapy for Lung Cancer. Cancer Discovery 1(1):44-53, 6/2011. e-Pub 4/2011.





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. Recent studies show >3 mL plasma recommended
  - 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.