METHOD ARTICLE

REVISED CRE: a cost effective and rapid approach for PCR-mediated concatenation of *KRAS* and *EGFR* exons [version 2; referees: 2 approved]

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Abstract

Molecular diagnostics has changed the way lung cancer patients are treated worldwide. Of several different testing methods available, PCR followed by directed sequencing and amplification refractory mutation system (ARMS) are the two most commonly used diagnostic methods worldwide to detect mutations at *KRAS* exon 2 and *EGFR* kinase domain exons 18-21 in lung cancer. Compared to ARMS, the PCR followed by directed sequencing approach is relatively inexpensive but more cumbersome to perform. Moreover, with a limiting amount of genomic DNA from clinical formalin-fixed, paraffin-embedded (FFPE) specimens or fine biopsies of lung tumors, multiple rounds of PCR and sequencing reactions often get challenging. Here, we report a cost-effective single multiplex-PCR based method, CRE (for **C**o-amplification of five *K**R**AS* and ***E**GFR* exons), followed by concatenation of the PCR product as a single linear fragment for direct sequencing. CRE is a robust protocol that can be adapted for routine use in clinical diagnostics with reduced variability, cost and turnaround time requiring a minimal amount of template DNA extracted from FFPE or fresh frozen tumor samples. As a proof of principle, CRE is able to detect the activating *EGFR* L858R and T790M *EGFR* mutations in lung cancer cell line and primary tumors.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED version 2 published 08 Mar 2016	✓ report	
version 1 published 23 Jun 2015	? report	✓ report

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REVISED **Amendments from Version 1**

We are particularly grateful to Reviewer 1 for describing the study as "a well conducted proof of principle report", and Reviewer 2 for their comments that, "The methods are well described and the test is of clinical relevance, particularly in settings with limited resources and without access to tumor next generation sequencing". Further, incorporating the suggestions made by the reviewers have contributed to an improved version of the manuscript. Specifically, we have, in the revised version:

In response to Reviewer 1

a) We have incorporated the suggestion of the reviewer by correcting the original submission in response their comments 1, 3, 4 and 7.

b) We have included the relevant references as pointed by the reviewer comment 2.

c) We have detailed our response to rest of the queries.

In response to Reviewer 2

d) We have incorporated the suggestion of the reviewer by correcting the original submission in response to both of their comments 1 and 2.

See referee reports

Introduction

The growing significance of identifying *EGFR* and *KRAS* mutations in lung cancer using molecular diagnostic approaches underlines the emphasis on the use of personalized medical care by physicians to help design optimal therapeutic regimens (Lynch *et al.*, 2004; Paez *et al.*, 2004; Pao *et al.*, 2004; Pao *et al.*, 2005a; Pao *et al.*, 2005b). While *EGFR* and *KRAS* mutations largely occur mutually exclusively in non-small cell lung cancer (NSCLC), and predict contrasting response rate to tyrosine-kinase inhibitors (TKI) (Chougule *et al.*, 2013; Fukuoka *et al.*, 2011; Ihle *et al.*, 2012; Lynch *et al.*, 2004; Mao *et al.*, 2010; Mok *et al.*, 2009), some recent studies, including ours, suggest co-occurrence of *EGFR* and *KRAS* mutations in the same patients, albeit at low frequency (Chougule *et al.*, 2014; Li *et al.*, 2014). While no direct evidence exists as yet, these studies may have implications for carrying out routine KRAS molecular testing along with EGFR mutations for precluding a patient with NSCLC from therapy with EGFR inhibitors, as approved for colorectal cancer (Lievre *et al.*, 2006). Such information is especially important for lung cancer patients at an advanced-stage, who are not candidates for surgical intervention—wherein biopsy specimens obtained through fine-needle aspiration (FNA) may represent the only opportunity to obtain tissue material for diagnosis and molecular diagnostic analysis.

EGFR mutations in NSCLC are characterized by approximately 39 unique mutations present across exons 18-21. Of these, most common are activating mutations, which account for approximately 90% of all *EGFR* mutations and are closely related to the efficacy of EGFR-TKIs. These activating mutations include point mutations G719S, T790M, L858R, and L861Q in exons 18, 20 and 21 respectively and in-frame deletions/insertions in exon 19 (Kosaka *et al.*, 2004). The most common mutations that result in an amino acid substitution at position 12 and 13 in *KRAS* are G12V and G13D (Choughule *et al.*, 2014). Several screening and target based methods are currently in use for to infer the *EGFR* and *KRAS* hot spot mutations, viz; PCR-RFLP (Restriction fragment length polymorphism), Amplification Refractory Mutation System (ARMS), PCR-Invader, TaqMan PCR, allele specific qPCR, high resolution melting analysis and ultra-deep pyrosequencing, SNaPshot analysis and co-amplification at lower denaturation temperature (COLD)-PCR (Angulo *et al.*, 2012; Borràs *et al.*, 2011; Ellison *et al.*, 2013; Santis *et al.*, 2011; van Eijk *et al.*, 2011; Zinsky *et al.*, 2010). Of these, direct sequencing is the most commonly used method worldwide (Yatabe *et al.*, 2015). However, a typical PCR reaction that precedes the sequencing step to amplify 4 *EGFR* and 1 *KRAS* exon(s) essentially consists of five rounds of independent PCR requiring separate aliquots of genomic DNA template for each reaction, followed by ten rounds of sequencing reactions. With a limited amount of genomic DNA from clinical FFPE specimens or fine biopsies of lung tumors, multiple rounds of PCR and sequencing reactions can often be challenging to perform.

In-frame concatenation or assembly of individually amplified exons from genomic DNA to generate a coding fragment has been described in earlier research, wherein the total number of PCR reactions corresponds to the number of exons to be concatenated (An *et al.*, 2007; Fedchenko *et al.*, 2013; Mitani *et al.*, 2004; Tuohy & Groden, 1998). Here, we describe a novel methodology to co-amplify all four *EGFR* exons 18-21 along with *KRAS* exon 2 in a single multiplex PCR followed by directional or ordered concatenation of the products in the form of a single linear fragment. This concatenated product can be used to detect mutations by direct sequencing, at a much reduced cost and duration, and with a much smaller amount of template.

Materials and methods

Samples

Genomic DNA was isolated from human NSCLC cell line NCI-H1975 and primary fresh frozen tumor tissue using QIAamp DNA blood mini kit (Qiagen). Genomic DNA from FFPE blocks was isolated using QIAamp DNA FFPE tissue kit (Qiagen) as per manufacturer's instructions. DNA concentration was determined by absorbance at 280 nm (NanoDrop 2000, Thermo Scientific).

Primer design

PCR primers were designed for *KRAS* exon 2 and *EGFR* exons 18-21. Supplementary Table S1 represents all the primers used for PCR amplifications. With the exception of the OAD176 and OAD152 primers, all internal primers contain an additional overhang of 15 nucleotides, such that the tail sequence of forward and reverse primers of two subsequent exons are complementary to each other to allow ordered and directional concatenation of *KRAS* and *EGFR* exons. The full length concatenated product of 915 bases was amplified using OAD176 and OAD152 primers.

Multiplex PCR of *KRAS* exon 2 and *EGFR* exons 18-21

Multiplex PCR (50 µl per reaction) was carried out in a single tube by using multiplex PCR kit (Qiagen) containing either 10 ng of genomic DNA from the NSCLC cell line or fresh frozen primary tumor, or 50 ng of genomic DNA from FFPE blocks with 0.2 µM each of the five primer pairs using Applied Biosystems Veriti 96-Well Thermal Cycler. PCR was carried out with initial hot-start denaturation at 95°C for 15 min, followed by 35 cycle of denaturation at 94°C for 30 seconds, annealing at 57°C for 90 seconds, polymerization at 72°C for 60 seconds, and final incubation for 30 min at 60°C. The multiplex PCR products were analyzed by agarose gel electrophoresis.

Concatenation of exons and sequencing analysis

For concatenation of *KRAS* exon 2 and *EGFR* exons 18-21, 2 µl of multiplex PCR product was used as template in a 50 µl PCR reaction containing 0.2 µM of each OAD176 and OAD152 primers. PCR was carried out in a Verity thermal cycler (Applied Biosystems) with an initial hot-start denaturation at 95°C for 15 min, followed by 35 cycle of denaturation at 94°C for 30 seconds, annealing at 57°C for 90 seconds, polymerization at 72°C for 60 seconds, and final incubation for 30 min at 60°C. Concatenated PCR product was analyzed by agarose gel electrophoresis. Sequencing of concatenated PCR products were performed by Sanger sequencing. Sequences were analyzed using Mutation Surveyor software V4.0.9 (Minton *et al.*, 2011).

Results

CRE (**C**o-amplification of ***KR****AS* and **E**GFR) exons is a cost-effective multiplex-PCR based method followed by concatenation of the PCR product as a single fragment for direct sequencing (Figure 1). It is a robust methodology to determine the mutation status of *KRAS* and *EGFR* with reduced variability, cost and turnaround time, requiring a minimal amount of template DNA extracted from FFPE or fresh frozen tumor samples.

CRE-based *KRAS-EGFR* concatenation from fresh frozen primary tumors and tumor-derived cell lines

Following CRE-based multiplex PCR of *KRAS* exon 2 and *EGFR* exons 18-21 with overlapping PCR bands (Figure 2A, lane 6), concatenation of the PCR product was performed with OAD176 and OAD152 primers using genomic DNA extracted from NCI-H1975 cells, a non-small-cell lung adenocarcinoma cell line. Concatenation PCR resulted in the enrichment of a concatenated product of

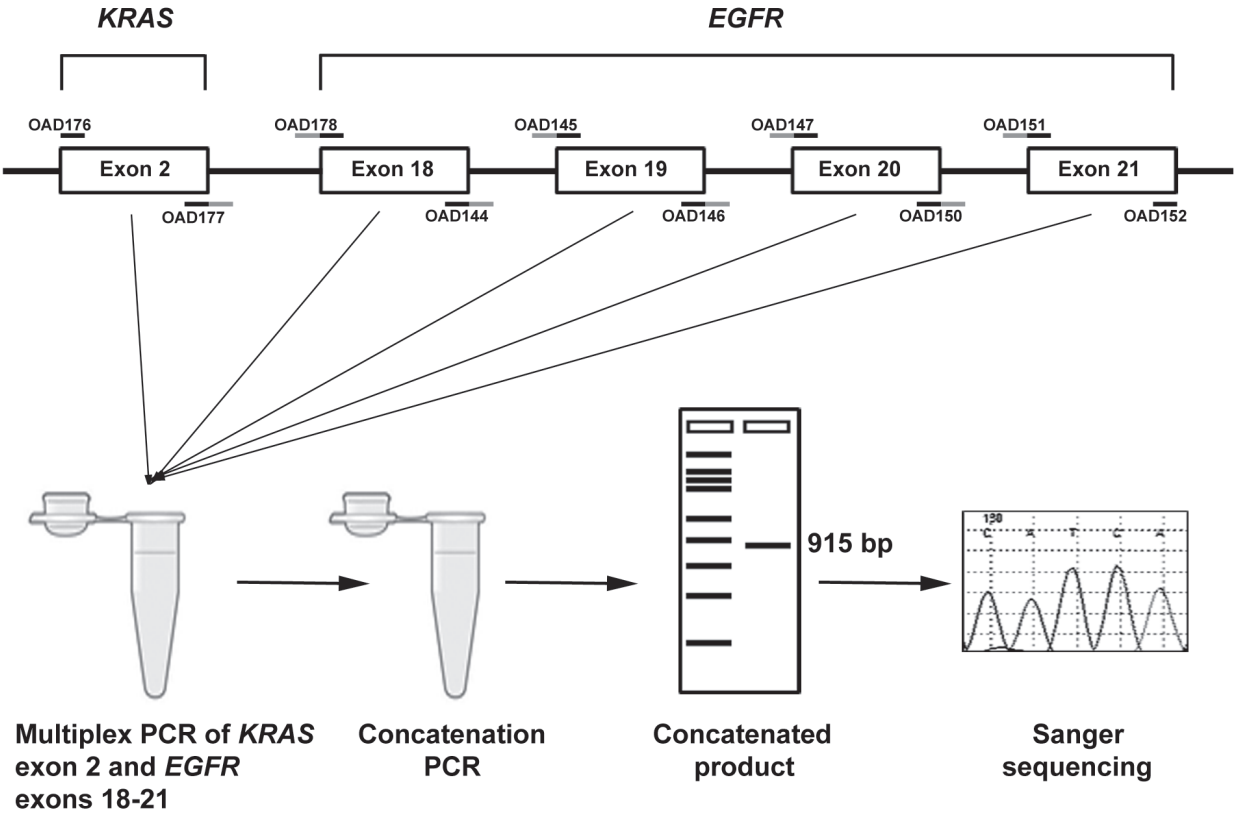

Figure 1. Schematic representation of CRE: Concatenation of *KRAS* and *EGFR* exons. The flowchart represents the workflow for CRE methodology. *KRAS* and *EGFR* primers are shown along with complementary tail overhangs that prime with consecutive exons in an ordered manner. 2 µl PCR products, amplified with a cocktail of primers, as shown and described in Supplementary Table S1, for *KRAS* and *EGFR* exons in a single multiplex reaction is transferred to a fresh tube and concatenated in a separate reaction using OAD 176 and OAD 152 primers. The concatenated product obtained is a single product of 915 bp with all individual exons amplified from multiplex PCR ligated together in an ordered manner as a single fragment. 2x sequencing using the forward primer OAD 176 and reverse primer OAD 152 of the concatenated product is adequate to scan the mutation status across all the *KRAS* and *EGFR* exons.

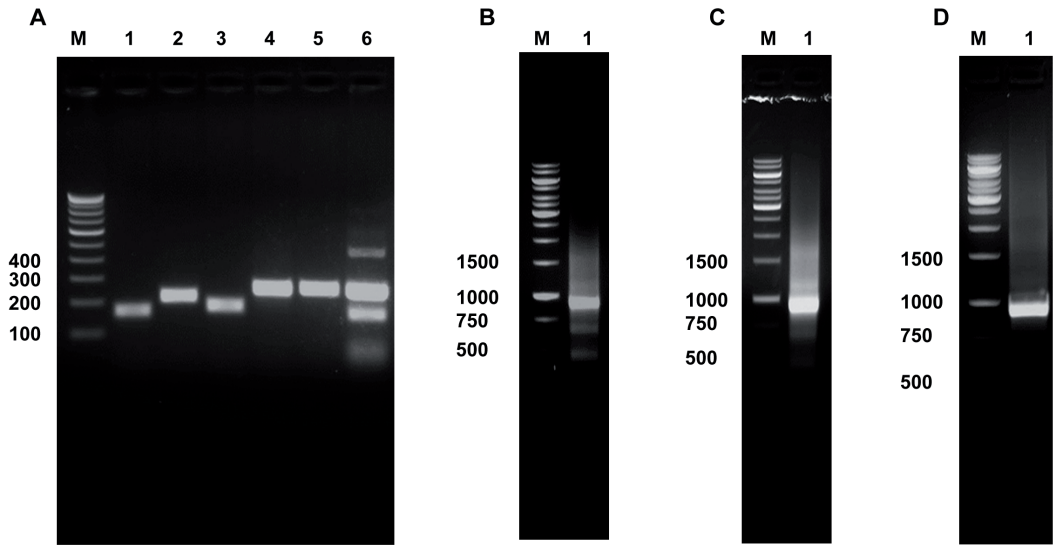

Figure 2. Multiplex PCR amplification and concatenation of *KRAS* and *EGFR* exons generates CRE product. Panel A. PCR amplification of *KRAS* and *EGFR* exons using NCI-H1975 genomic DNA: Lane 1, *KRAS* exon 2 (151 bp) amplified with OAD176 and OAD177; Lane 2, *EGFR* exon 18 (209 bp) amplified with OAD 178 and OAD 144; Lane 3, *EGFR* exon 19 (178 bp) amplified with OAD 145 and OAD 146; Lane 4, *EGFR* exon 20 (246 bp) amplified with OAD 147 and OAD 150; Lane 5, *EGFR* exon 21 (251 bp) amplified with OAD 151 and OAD 152; Lane 6, Multiplex PCR of *KRAS* exon 2 and *EGFR* exons 18-21 with cocktail of primers used in Lanes 1–5. Concatenated *KRAS* and *EGFR* (CRE) product of ~915 bp amplified with OAD 176 and OAD 152 using multiplex PCR product as template derived from NCI-H1975 genomic DNA (shown in **Panel B**, Lane 2); derived from fresh frozen primary tumor genomic DNA (shown in **Panel C**, Lane 2); using tumor genomic DNA extracted from FFPE block (shown in **Panel D**, Lane 2).

about 915 base pairs (Figure 2B). This concatenated, gel purified PCR product of 915 base pair was used for Sanger sequencing. Sequencing analysis of the concatenated PCR product confirmed concatenation as a single fragment (Figure 3) along with the presence of *EGFR* T790M and L585R mutations in NCI-H1975 cells (Supplementary Figure S1). A similar concatenation of a 915 bp single fragment was performed with genomic DNA extracted from fresh frozen tumor cells (Figure 2C).

Dataset 1. Raw gel electrophoresis images for Figure 2: Multiplex PCR amplification and concatenation of *KRAS* and *EGFR* exons generates CRE product

http://dx.doi.org/10.5256/f1000research.6663.d50236

Zip file contains 4 files: Raw image for Figure 2A, Raw image for Figure 2B, Raw image for Figure 2C, Raw image for Figure 2D.

Panel A. PCR amplification of *KRAS* and *EGFR* exons using NCI-H1975 genomic DNA: Lane 1, *KRAS* exon 2 (151 bp) amplified with OAD176 and OAD177; Lane 2, *EGFR* exon 18 (209 bp) amplified with OAD 178 and OAD 144; Lane 3, *EGFR* exon 19 (178 bp) amplified with OAD 145 and OAD 146; Lane 4, *EGFR* exon 20 (246 bp) amplified with OAD 147 and OAD 150; Lane 5, *EGFR* exon 21 (251 bp) amplified with OAD 151 and OAD 152; Lane 6, Multiplex PCR of *KRAS* exon 2 and *EGFR* exons 18–21 with cocktail of primers used in Lanes 1–5.

Concatenated *KRAS* and *EGFR* (CRE) product of ~915 bp amplified with OAD 176 and OAD 152 using multiplex PCR product as template derived from NCI-H1975 genomic DNA (shown in **Panel B**, Lane 2); derived from fresh frozen primary tumor genomic DNA (shown in **Panel C**, Lane 2); using tumor genomic DNA extracted from FFPE block (shown in **Panel D**, Lane 2) (Ramteke *et al.*, 2015a).

Dataset 2. Sequencing traces for Figure 3: Full length sequencing of the CRE product

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Zip file contains 4 files: Sequencing trace for Figure 3A .ab1, Sequencing trace for Figure 3B .ab1, Sequencing trace for Figure 3C .ab1 and Sequencing trace for Figure 3D .ab1.

Reverse complements of the forward sequencing reads of the 915 bp *KRAS-EGFR* concatenated product are displayed as generated by Mutation Surveyor V4.0.9. Panel A displays 15 nucleotide junction region flanked by *KRAS* exon 2 and *EGFR* exon 18 sequence; Panel B displays 15 nucleotide junction region flanked by *EGFR* exons 18 and 19; Panel C displays 15 nucleotide junction region flanked by *EGFR* exons 19 and 20; and displays 15 nucleotide junction region flanked by *EGFR* exons 20 and 21 is shown in Panel D (Ramteke *et al.*, 2015b).

CRE-based *KRAS-EGFR* concatenation from paraffin-embedded clinical cancer specimens

The amount of genomic DNA obtained from FFPE tissue is always limiting and thus there is a substantial need to develop a technique with a limited amount of starting DNA as a template for mutation detection. CRE demonstrates the ability to co-amplify all five exons (*KRAS* exon 2 and *EGFR* exon 18-21) in a single multiplex PCR reaction with a limited amount of starting template DNA followed by the enrichment of concatenated product (Figure 2D) by concatenation PCR using first multiplex PCR product as a template.The concatenated product confirmed *EGFR* L858R mutation in the FFPE tissues (Supplementary Figure S2), as reported earlier (Choughule *et al.*, 2014). Thus our CRE method can be routinely used for the mutational analysis of *KRAS* and *EGFR* genes.

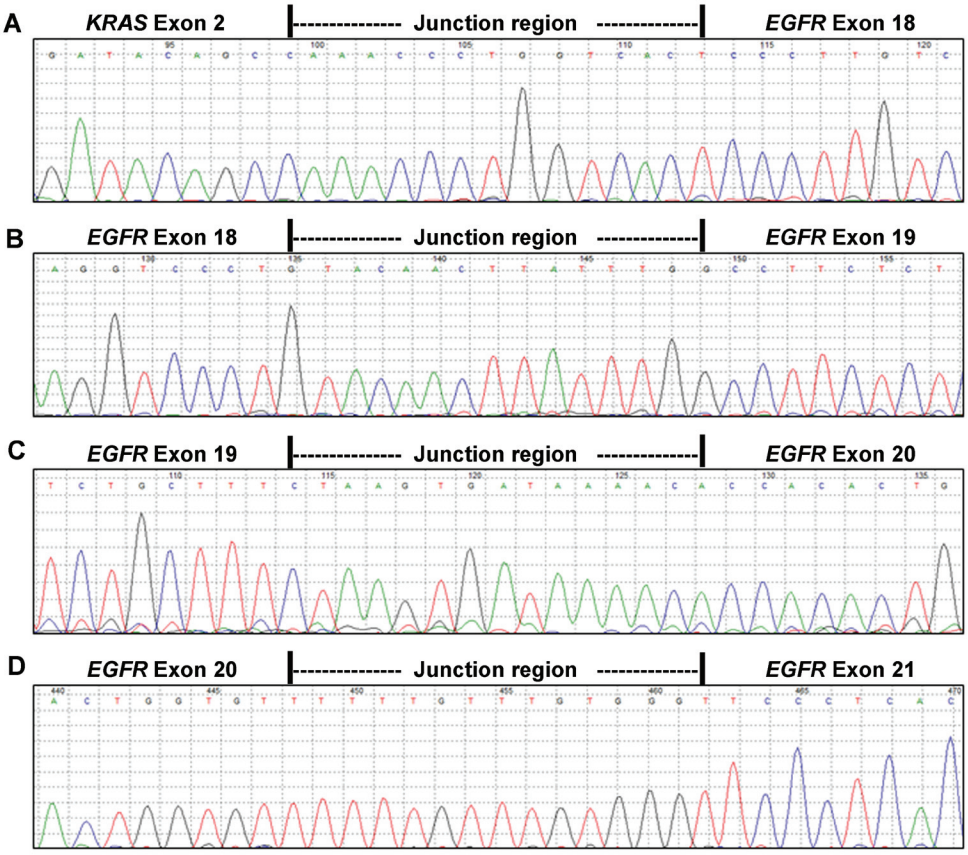

Figure 3. Full length sequencing of the CRE product. Reverse complements of the forward sequencing reads of the 915 bp *KRAS-EGFR* concatenated product are displayed as generated by Mutation Surveyor V4.0.9. **Panel A** displays 15 nucleotide junction region flanked by *KRAS* exon 2 and *EGFR* exon 18 sequence; **Panel B** displays 15 nucleotide junction region flanked by *EGFR* exons 18 and 19; **Panel C** displays 15 nucleotide junction region flanked by *EGFR* exons 19 and 20; and. displays 15 nucleotide junction region flanked by *EGFR* exons 20 and 21 is shown in **Panel D**.

Discussion

CRE is a novel, simple and effective strategy to concatenate multiple amplicons obtained from a multiplex PCR, using primers with overlapping complementary overhangs. Compared to ARMS, and other genotyping technologies, CRE is relatively inexpensive with faster turnaround time involving lesser amount of template genomic DNA.

Using CRE, *in vitro* tandem reconstitution of *KRAS* exon 2 with *EGFR* exons 18-21 can be effectively performed to generate a concatenated single PCR product of 915 bp, as a template for sequencing. Most commercially-available allele-specific and genotyping technologies are restricted by their ability to probe only for eight out of the approximately 39 known commonly occurring *EGFR* and *KRAS* activating mutations. However, growing clinical data on the less common mutations are now emerging to fully inform their predictable outcomes on EGFR TKIs (Lohinai *et al.*, 2015; Yang *et al.*, 2012). Currently available methodologies, if extended to genotype all known 39 mutations would not only be cost-prohibitive but challenging to perform due to a limiting amount of template genomic DNA available from clinical cancer specimens that are mostly available in the form of formalin-fixed, paraffin-embedded (FFPE) tissue. While a directed sequencing approach –classical or next-generation sequencing (NGS) -based—can determine a whole spectrum of rare and co-occurring mutations in an individual, the question of template genomic DNA availability still remains. CRE circumvents the issue of a limiting amount of template genomic DNA with increased affordability by multiplexing PCR for all exons to a single reaction and concatenating the PCR product as a single fragment, thereby further reducing the cost of multiple sequencing reactions.

In this era of genome sequencing, applicability of the CRE strategy could be of immense significance to effectively reduce the cost and turnaround time taken to determine the mutational status across the whole *KRAS* exon 2 and *EGFR* kinase domain exons. As the limitation of the CRE strategy is defined by the sensitivity and resolution of the sequencing methodology adopted, concatenated *EGFR* and *KRAS* PCR products from multiple individuals—each tagged with unique bar code sequence—can be pooled and deep-sequenced using a NGS platform. The CRE strategy described here can reduce the labor and cost of performing individual PCR for all exons for

each patient and effectively circumvent the noise due to variation in normalization for equimolar pooling of exons within the same sample at a resolution of single base. Additionally, the current version of CRE is limited by exclusion of fewer number of exons of *EGFR* and *KRAS*. Inclusion of known extracellular *EGFR* and *KRAS* exon 3 codon 61 mutation may help to immediately expand the scope of its application to other cancers, such as glioblastoma.

Data availability

F1000Research: Dataset 1. Raw gel electrophoresis images for Figure 2: Multiplex PCR amplification and concatenation of *KRAS* and *EGFR* exons generates CRE product, 10.5256/f1000research.6663.d50236

F1000Research: Dataset 2. Sequencing traces for Figure 3: Full length sequencing of the CRE product, 10.5256/f1000research.6663.d50237

F1000Research: Dataset 3. Sequencing traces for Figure S1: Detection of *EGFR* T790M and L858R mutations from NCI-H1975 CRE product, 10.5256/f1000research.6663.d50238

F1000Research: Dataset 4. Sequencing trace for Figure S2: Detection of *EGFR* L858R mutation in a CRE product derived from FFPE primary tumor sample, 10.5256/f1000research.6663.d50239

Author contributions

M.P.R. and K.J.P. contributed equally to this work. M.P.R., K.J.P, K.P. and A.D. conceived and designed the experiments. M.P.R., K.J.P, M.G., M.V., and K.K. performed the experiments. M.P.R., K.J.P. and A.D. analyzed the data. A.C. and K.P. contributed reagents/materials/analysis tools. M.P.R., K.J.P. and A.D. wrote the paper.

Competing interests

The authors declared no competing interests.

Grant information

A.D. is supported by an Intermediate Fellowship from the Wellcome Trust/DBT India Alliance (IA/I/11/2500278), by a grant from DBT (BT/PR2372/AGR/36/696/2011), and intramural grants (IRB project 55, 88, 92, 107, 108, 116).

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary materials

Supplementary Table S1. Sequences with underline denote priming region of primer. Sequences in italics indicate extra 15 nucleotide tail sequences (junction region). Sequences in bold denotes complementary region between reverse primer of one exon with forward primers of successive exon. 5′ and 3′ represents forward and reverse primer respectively.

Primer	Primer information	Amplicon size (bp)	Sequences
OAD176	5′ *KRAS* exon 2	151	C C TTATGTGTGACAT GTTCTAATATAGTCA C
OAD177	3′ *KRAS* exon 2		**ACACAGAGACAAGGG***AGTGACCAGGGTTTG* GCTGTATCGTCAA GGC AC
OAD178	5′ *EGFR* exon 18	209	*CAAACCCTGGTCACT* **CCCTT GT CTCTGT GT**TCTTGTC C C C CC C AG
OAD144	3′ *EGFR* exon 18		**CTATGACAGAGAGAGAAGG***CCAAATAAGTTGTAC* AGGGACC TTACC TTA
OAD145	5′ *EGFR* exon 19	178	*GTACAACTTATTTGG***CCTTCTCTCTCTGTCATAG**GGACTCTGGAT
OAD146	3′ *EGFR* exon 19		**GGCACGTCAGTGTGG***TGTTTTATCACTTAG* AAAGC AGA AAC TCAC
OAD147	5′ *EGFR* exon 20	246	*CTAAGTGATAAAACA* **CCACACT GAC GT GCC**TCTC C C TCC C TCC AG
OAD150	3′ *EGFR* exon 20		**CCCTGCTGTGAGGGA***ACCCACAAACAAAAA*ACACCAGTTGAGCAG
OAD151	5′ *EGFR* exon 21	251	*TTTTTGTTTGTGGGT* **TCCCTCACA GCA GG G**TCTTC TCTGTTTCA G
OAD152	3′ *EGFR* exon 21		TGGTC C C TGGTGTC A GGAA

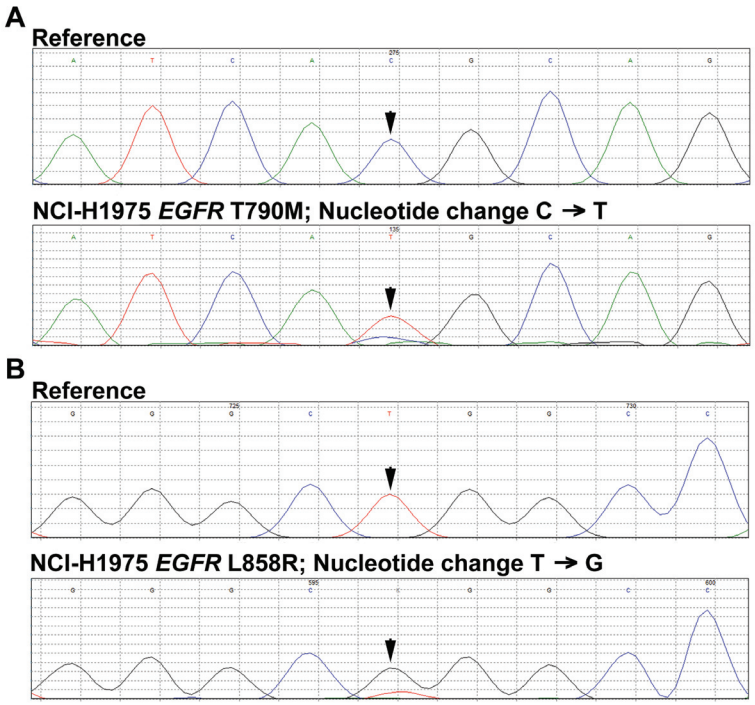

Figure S1. Detection of *EGFR* T790M and L858R mutations from NCI-H1975 CRE product. Reverse complements of the forward sequencing reads of the 915 bp CRE product using genomic DNA extracted from NCI-H1975 cells are displayed as generated by Mutation Surveyor. **Panel A**: The arrow indicates expected location of the wild-type and T790M mutant allele peak. **Panel B**: The arrow indicates expected location of the wild-type and L858R mutant allele peak.

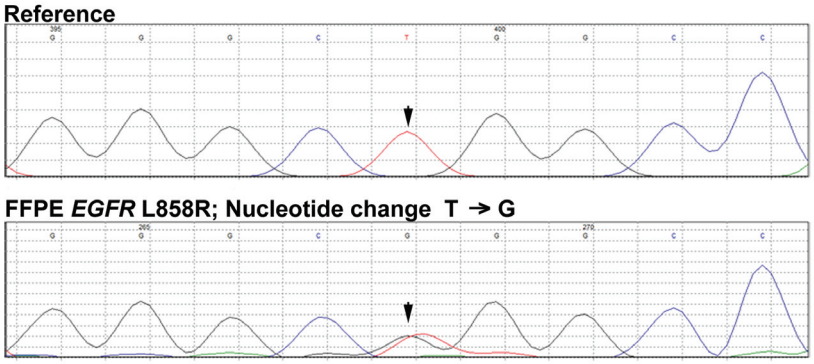

Figure S2. Detection of *EGFR* L858R mutation in a CRE product derived from FFPE primary tumor sample. Reverse complements of the forward sequencing reads of the 915 bp CRE product using genomic DNA extracted from FFPE primary tumor are displayed are displayed as generated by Mutation Surveyor. The arrow indicates expected location of the wild-type and L858R mutant allele peak.

Dataset 3. Sequencing traces for Figure S1: Detection of *EGFR* T790M and L858R mutations from NCI-H1975 CRE product

http://dx.doi.org/10.5256/f1000research.6663.d50238

Zip file contains 2 files: Sequencing trace for Figure S1B .ab1 and Sequencing trace for Figure S1A .ab1

Reverse complements of the forward sequencing reads of the 915 bp CRE product using genomic DNA extracted from NCI-H1975 cells are displayed as generated by Mutation Surveyor. Panel A: The arrow indicates expected location of the wild-type and T790M mutant allele peak. Panel B: The arrow indicates expected location of the wild-type and L858R mutant allele peak (Ramteke *et al.*, 2015c).

Dataset 4. Sequencing trace for Figure S2: Detection of *EGFR* L858R mutation in a CRE product derived from FFPE primary tumor sample

http://dx.doi.org/10.5256/f1000research.6663.d50239

Reverse complements of the forward sequencing reads of the 915 bp CRE product using genomic DNA extracted from FFPE primary tumor are displayed are displayed as generated by Mutation Surveyor. The arrow indicates expected location of the wild-type and L858R mutant allele peak (Ramteke *et al.*, 2015d).

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Data Source

Ramteke MP, Patel KJ, Godbole M, *et al.*: **Dataset 2 in CRE: a cost-effective and rapid approach for PCR-mediated concatenation of *KRAS* and *EGFR* exons.** *F1000Research.* 2015b.
Data Source

Ramteke MP, Patel KJ, Godbole M, *et al.*: **Dataset 3 in CRE: a cost-effective and rapid approach for PCR-mediated concatenation of *KRAS* and *EGFR* exons.** *F1000Research.* 2015c.
Data Source

Ramteke MP, Patel KJ, Godbole M, *et al.*: **Dataset 4 in CRE: a cost-effective and rapid approach for PCR-mediated concatenation of *KRAS* and *EGFR* exons.** *F1000Research.* 2015d.
Data Source

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Open Peer Review

Current Referee Status:

Version 2

Referee Report 05 April 2016

doi:10.5256/f1000research.8838.r9437

Chandan Kumar
Department of Pathology, University of Michigan, Ann Arbor, MI, USA

The authors have provided satisfactory responses to all the concerns. Within the limits of sensitivity of Sanger sequencing, and currently limited to the the exons examined, the relatively simple, cost effective assay system presented here is a practical solution to an important clinical question in resource limited environment.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 24 February 2016

doi:10.5256/f1000research.7157.r9740

Bob T. Li
Thoracic Oncology Service, Division of Solid Tumor Oncology, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

In the age of precision medicine with an expanding number of oncogenic drivers in lung cancers that may be treated with targeted agents, multiplexed genomic testing is increasingly important in clinical practice. The study by Ramteke et al. describes a rapid and relatively inexpensive multiplexed test for EGFR and KRAS mutations. The methods are well described and the test is of clinical relevance, particularly in settings with limited resources and without access to tumor next generation sequencing. I recommend making the following minor revisions:

1. In the introduction, it is incorrect to suggest that the reason for KRAS testing in lung cancers is to preclude patients from EGFR inhibitors. The rationale for EGFR inhibitors in lung cancers is very different to that of colorectal cancers, as activating EGFR mutations in lung cancers predict response to EGFR TKIs. However, it is still important to test all lung cancers for KRAS mutations as it is a common oncogenic driver occuring in over 25% of lung adenocarcinomas. Being a driver KRAS is highly unlikely to co-exist with other actionable drivers, therefore once KRAS is found one

could justify that further genomic testing for other drivers is not necessary, especially in a resource limited setting.

2. It should be acknowledged that the authors' CRE method will not capture all KRAS mutations, especially KRAS mutations in exon 3 codon 61. However, the ability to capture the majority of KRAS and EGFR mutations in one single inexpensive test is still of value for patients with lung cancers.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response (*Member of the F1000 Faculty*) 24 Feb 2016

Amit Dutt, ACTREC, Tata Memorial Center,, India

We sincerely thank the reviewer for the elaborate and detailed constructive review. In particular, we are grateful to the reviewer for describing the study as *"a well conducted proof of principle report".* We hope the reviewer will find the improved version of the manuscript acceptable, without reservation. Our response to specific concerns are as follows-

Referee's comment 1: *Introduction, "These studies have direct implications for carrying out routine KRAS molecular testing along with EGFR mutations for precluding a patient with NSCLC from therapy with EGFR inhibitors, as approved for colorectal cancer (Lievre et al., 2006)." Can the authors cite any reference wherein NSCLC patients are precluded from EGFR inhibitor therapy if they harbor KRAS mutations. The reference cited here is specific to colorectal cancer.*

Author's response: *EGFR* and *KRAS* mutations occur mutually exclusive in NSCLC, which suggests functional redundancy, however they predict contrasting response rates to tyrosine-kinase inhibitors (TKI). While *EGFR* mutation predicts longer progression-free survival rate, adverse prognosis is associated with patients harboring *KRAS* mutations. Thus, the recently reported co-occurrence of *KRAS* and *EGFR* activating mutations in 30 of 5125 patients, along with our study of co-occurrence of *KRAS* and *EGFR* activating mutations in 3 of 86 patients, raises questions about the relative value of *EGFR* and *KRAS* mutation status as predictors of outcome in NSCLC. As the reviewer may agree these studies may have obvious implications for routine *KRAS* testing in this disease, potentially precluding EGFR TKI therapy from some patients, similar to current practice in colorectal cancer. However, their direct mention in NSCLC is speculative.

Thus, in principle, we fully agree with the reviewer that no direct evidence exists to preclude EGFR inhibitor therapy among patients co-harboring *EGFR* and *KRAS* mutation. In accordance with the reviewer's suggestion we have revised the text to reflect the speculative implication of our methodology in NSCLC. Our modified text reads as follows:
"….While no evidence exists as yet, these studies may have implications for carrying out routine KRAS molecular testing along with EGFR mutations for precluding a patient with NSCLC from therapy with EGFR inhibitors, as approved for colorectal cancer (Lievre et al., 2006)…."

Referee's comment 2: *Introduction, "Of these direct sequencing is the most commonly used method worldwide". Is this a personal opinion or there is a reference to support this. Should be cited.*

Author's response: We thank the reviewer for pointing the omission. A relevant citation has been added. However, as our citation in manuscript is likely to be incomplete to summarize the field, some additional studies are mentioned below:

Yatabe, Yasushi, et al. "EGFR Mutation Testing Practices within the Asia Pacific Region: Results of a Multicenter Diagnostic Survey." *Journal of Thoracic Oncology* 10.3 (2015): 438;

Angulo, Bárbara, et al. "A comparison of EGFR mutation testing methods in lung carcinoma: direct sequencing, real-time PCR and immunohistochemistry." *PLoS One* 7.8 (2012): e43842;

Ellison, Gillian, et al. "EGFR mutation testing in lung cancer: a review of available methods and their use for analysis of tumour tissue and cytology samples." *Journal of clinical pathology* 66.2 (2013): 79-89;

Cappuzzo, Federico. "Methods for EGFR Mutation Testing." *Guide to Targeted Therapies: EGFR mutations in NSCLC*. Springer International Publishing, 2014. 19-24.

Referee's comment 3: *Introduction, "concatenation or assembly of individually amplified exons from genomic DNA to generate a cDNA fragment has been described in earlier research". cDNA specifically refers to complementary DNA derived from RNA through reverse transcriptase. Genomic PCR cannot be said to be used to generate cDNA fragment. I suspect this erroneous phrasing is picked up from a previous reference, but its probably good to not perpetuate the error.*

Author's response: We agree and thank the reviewer for bringing it our attention. Our modified text reads as follows:
"….concatenation or assembly of individually amplified exons from genomic DNA to generate a coding fragment has been described in earlier research…".

Referee's comment 4: *Introduction, "Here, we describe a novel methodology to co-amplify all four EGFR exons 18–21 along with KRAS exon 2 in a single multiplex PCR". It's more like a novel application of a well described methodology supported by several previous references. The novelty, albeit rather incremental, is in combining exons from two different genes, using previously described approach. Should be stated as such.*

Author's response: As suggested by the reviewer, we have dropped the term "novel". Our modified text reads as follows:
"….Here, we describe a methodology to co-amplify all four *EGFR* exons 18–21 along with *KRAS* exon 2 in a single multiplex PCR…."

Referee's comment 5: *The big appeal of the study is that it affords use of a minimal amount of FFPE sample. Please specify the amount of FFPE material used and yield of DNA to convey an idea of how little/much sample is needed to carry out this analysis.*

Author's response: As mentioned in the methodology section subtitled, "Multiplex PCR of *KRAS* exon 2 and *EGFR* exons 18-21", multiplex PCR (50µl per reaction) was carried out in a single tube by using multiplex PCR kit (Qiagen) containing either 10 ng of genomic DNA from the NSCLC cell line or fresh frozen primary tumor, or 50 ng of genomic DNA from FFPE blocks. Furthermore, as mentioned under the methodology section subtitled, "Concatenation of exons and sequencing

analysis", 2 µl of multiplex PCR product was used as template in a 50 µl PCR reaction for concatenation.

Referee's comment 6: *The important consideration of sensitivity has not been addressed. This could be easily tested by assaying a serial dilution of known mutated cell line/FFPE DNA spiked in a wild-type background sample. This will add value to the study.*

Author's response: As mentioned under the last paragraph of the discussion section, "….As the limitation of the CRE strategy is defined by the sensitivity and resolution of the sequencing methodology adopted…" – which in this study has been Sanger Sequencing, but could significantly vary if advanced contemporary sequencing methodologies are adopted. However, as the sensitivity of PCR followed by directed Sanger Sequencing is well established from FFPE samples and mutated cell line, we humbly differ from the reviewer that admixture experiment would add additional information.

Referee's comment 7: *Addition of KRAS codon 61 should be considered as well. Or the difficulty in scaling up should be discussed. How difficult is it to add additional exons.*

Author's response: *KRAS* codon 12 is mutated at a frequency of 25-50% in Caucasian population and 5-15% among East Asians. In a recent study we reported 18.6% *KRAS* codon 12 among Indian population (n=86)-- (Choughule *et al.*, 2014). Given that *KRAS* codon 61 mutation exist at frequency < 1%; and, that none were found in our study in 86 patients, we decided to not include *KRAS* codon 61 mutation in this study to only present the proof of principle of the CRE methodology. However, we do agree with the reviewer about the significance of *KRAS* codon 61 mutation, and do hope to include it along with other known activating mutations in NSCLC in an improved version of CRE.

However, to reflect the pertinent suggestion made by the reviewer we have modified our discussion to read as follows:
"…Additionally, the current version of CRE is limited by exclusion of fewer number of exons of *EGFR* and *KRAS*. Inclusion of known extracellular *EGFR* and *KRAS* exon 3 codon 61 mutation may help to immediately expand the scope of its application to other cancers, such as glioblastoma ."

Referee's comment 8: *The concatenated PCR product may be amenable to Pyrosequencing to improve sensitivity of detection (particularly in case of low tumor content, low clonality of mutations as is expected in case of dynamically evolving tumors). This should be attempted/ discussed.*

Author's response: We fully agree with the reviewer's insights that CRE can be utilized at high throughput mode to determine complete spectrum of *EGFR* and *KRAS* mutations using targeted next generation sequencing. Consistent with the reviewer's suggestion the last paragraph of the discussion section, "… the limitation of the CRE strategy is defined by the sensitivity and resolution of the sequencing methodology adopted, concatenated *EGFR* and *KRAS* PCR products from multiple individuals—each tagged with unique bar code sequence—can be pooled and deep-sequenced using a NGS platform. The CRE strategy described here can reduce the labor and cost of performing individual PCR for all exons for each patient and effectively circumvent the noise due to variation in normalization for equimolar pooling of exons within the same sample at a resolution of single base."

Referee's comment 9: *A direct comparison with the standard technique(s) currently used to test these mutations- in terms of amount of starting material needed, sensitivity of detection, time, and cost will help the argument of the new approach as a superior option.*

Author's response: As detailed in the manuscript, this proof of principle study introduces CRE as a methodology involving single multiplex-PCR followed by concatenation of the PCR product as one linear fragment for direct sequencing, as opposed to 5 rounds of PCR reaction followed by 10 rounds of sequencing reactions. A systematic comparative analysis is currently underway at our center using clinical cancer specimens for CRE compared to Sanger sequencing based methodology; SNaPShot PCR; Cobas system; Mass spec genotyping on a larger cohort sample, beyond the scope of this manuscript. Hence, we express our inability to include analysis from this ongoing study at this early on stage.

Competing Interests: No competing interests were disclosed.No competing interests were disclosed.

Author Response (*Member of the F1000 Faculty*) 24 Feb 2016

Amit Dutt, ACTREC, Tata Memorial Center,, India

We sincerely thank reviewer for approving our submission. We are particularly grateful to the reviewer for describing the study as, *"The methods are well described and the test is of clinical relevance, particularly in settings with limited resources and without access to tumor next generation sequencing".* The suggestions made by the reviewers have contributed to an improved version of the manuscript. Specifically, we have, in the revised version:

Referee's comment 1: *In the introduction, it is incorrect to suggest that the reason for KRAS testing in lung cancers is to preclude patients from EGFR inhibitors. The rationale for EGFR inhibitors in lung cancers is very different to that of colorectal cancers, as activating EGFR mutations in lung cancers predict response to EGFR TKIs. However, it is still important to test all lung cancers for KRAS mutations as it is a common oncogenic driver occuring in over 25% of lung adenocarcinomas. Being a driver KRAS is highly unlikely to co-exist with other actionable drivers, therefore once KRAS is found one could justify that further genomic testing for other drivers is not necessary, especially in a resource limited setting.*

Author's response: As described in or response to Reviewer 1's first comment, we agree we with the reviewer that no direct evidence exists to preclude EGFR inhibitor therapy among patients co-harboring *EGFR* and *KRAS* mutation. In accordance with the reviewer's suggestion we have revised the text to reflect the speculative implication of our methodology in NSCLC. Our modified text reads as follows:
"….While no evidence exists as yet, these studies may have implications for carrying out routine KRAS molecular testing along with EGFR mutations for precluding a patient with NSCLC from therapy with EGFR inhibitors, as approved for colorectal cancer (Lievre et al., 2006)…."

Referee's comment 2: *It should be acknowledged that the authors' CRE method will not capture all KRAS mutations, especially KRAS mutations in exon 3 codon 61. However, the ability to capture the majority of KRAS and EGFR mutations in one single inexpensive test is still of value for patients with lung cancers.*

Author's response: As described in our response to Reviewer 1's comment 7, we agree with the reviewer about the significance of *KRAS* codon 61 mutation, and do hope to include it along with other known activating mutations in NSCLC. However, to reflect the pertinent suggestion made by the reviewer we have modified our discussion to read as follows:
"…Additionally, the current version of CRE is limited by exclusion of fewer number of exons of *EGFR* and *KRAS*. Inclusion of known extracellular *EGFR* and *KRAS* exon 3 codon 61 mutation may help to immediately expand the scope of its application to other cancers, such as glioblastoma ."

Competing Interests: No competing interests were disclosed.No competing interests were disclosed.

Referee Report 31 July 2015

doi:10.5256/f1000research.7157.r9749

Chandan Kumar
Department of Pathology, University of Michigan, Ann Arbor, MI, USA

This is a well conducted proof of principle report that uses the approach of concatenated PCR of exons from genomic DNA to combine exons of two different, clinically relevant genes KRAS and EGFR, to address a clinically relevant question in a resource limiting setting. The assay design and data are provided in sufficient details that any researchers may be able to attempt to carry out similar analyses.

There are some comments and suggestions to improve the quality of the manuscript or follow up analyses:

1. **Introduction**, *"These studies have direct implications for carrying out routine KRAS molecular testing along with EGFR mutations for precluding a patient with NSCLC from therapy with EGFR inhibitors, as approved for colorectal cancer (Lievre et al., 2006)."*

   Can the authors cite any reference wherein NSCLC patients are precluded from EGFR inhibitor therapy if they harbor KRAS mutations. The reference cited here is specific to colorectal cancer.

2. **Introduction**, *"Of these direct sequencing is the most commonly used method worldwide".*

   Is this a personal opinion or there is a reference to support this. Should be cited.

3. **Introduction**, *"concatenation or assembly of individually amplified exons from genomic DNA to generate a cDNA fragment has been described in earlier research".*

   cDNA specifically refers to complementary DNA derived from RNA through reverse transcriptase. Genomic PCR cannot be said to be used to generate cDNA fragment. I suspect this erroneous phrasing is picked up from a previous reference, but its probably good to not perpetuate the error.

4. **Introduction**, *"Here, we describe a novel methodology to co-amplify all four EGFR exons 18–21 along with KRAS exon 2 in a single multiplex PCR".*

It's more like a novel application of a well described methodology supported by several previous references. The novelty, albeit rather incremental, is in combining exons from two different genes, using previously described approach. Should be stated as such.

5. The big appeal of the study is that it affords use of a minimal amount of FFPE sample. Please specify the amount of FFPE material used and yield of DNA to convey an idea of how little/much sample is needed to carry out this analysis.

6. The important consideration of sensitivity has not been addressed. This could be easily tested by assaying a serial dilution of known mutated cell line/FFPE DNA spiked in a wild-type background sample. This will add value to the study.

7. Addition of KRAS codon 61 should be considered as well. Or the difficulty in scaling up should be discussed. How difficult is it to add additional exons.

8. The concatenated PCR product may be amenable to Pyrosequencing to improve sensitivity of detection (particularly in case of low tumor content, low clonality of mutations as is expected in case of dynamically evolving tumors). This should be attempted/ discussed.

9. A direct comparison with the standard technique(s) currently used to test these mutations- in terms of amount of starting material needed, sensitivity of detection, time, and cost will help the argument of the new approach as a superior option.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 31 Jul 2015

Amit Dutt, ACTREC, Tata Memorial Center,, India

We thank the reviewer for describing the study as “a well conducted proof of principle report”, and for sharing elaborate comments and suggestions that has significantly improved the quality of the manuscript. Our response to specific concerns are as follows:

1. **Referee’s comments:** *Introduction, “These studies have direct implications for carrying out routine KRAS molecular testing along with EGFR mutations for precluding a patient with NSCLC from therapy with EGFR inhibitors, as approved for colorectal cancer (Lièvre et al., 2006).” Can the authors cite any reference wherein NSCLC patients are precluded from EGFR inhibitor therapy if they harbor KRAS mutations. The reference cited here is specific to colorectal cancer.*

   Author’s response: *EGFR* and *KRAS* mutations occur mutually exclusive in NSCLC, which suggests functional redundancy. However, they predict contrasting response rate to tyrosine-kinase inhibitors (TKI). While *EGFR* mutation predicts longer progression-free survival rate, adverse prognosis is associated with patients harboring *KRAS* mutations. Thus, the recently reported co-occurrence of *KRAS* and *EGFR* activating mutations in 30 of 5125 patients, along with our study of co-occurrence of *KRAS* and *EGFR* activating mutations in 3 of 86 patients, raises a clinical concern about the relative value of *EGFR* and

KRAS mutation status as predictors of outcome in NSCLC. As the reviewer may agree these studies may have obvious implications for routine *KRAS* testing in this disease, potentially precluding EGFR TKI therapy from some patients, similar to current practice in colorectal cancer.

In principle, we fully agree with the reviewer that no direct evidence exist to preclude EGFR inhibitor therapy among patients co-harboring *EGFR* and *KRAS* mutation. In accordance with the reviewer's suggestion we have revised the text to reflect the speculative implication of our methodology in NSCLC. Our modified text reads as follows:

"….While no evidence exists as yet, these studies may have implications for carrying out routine KRAS molecular testing along with EGFR mutations for precluding a patient with NSCLC from therapy with EGFR inhibitors, as approved for colorectal cancer (Lièvre et al., 2006)…."

2. **Referee's comments:** *Introduction, "Of these direct sequencing is the most commonly used method worldwide". Is this a personal opinion or there is a reference to support this. Should be cited.*

   Author's response: We thank the reviewer for pointing the omission. A relevant citation has been added. However, as our citation in manuscript is likely to be incomplete to summarize the field, some additional studies are mentioned below:

   Yatabe, Yasushi, et al. "EGFR Mutation Testing Practices within the Asia Pacific Region: Results of a Multicenter Diagnostic Survey." *Journal of Thoracic Oncology* 10.3 (2015): 438;

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generate a coding fragment has been described in earlier research…".

4. **Referee's comments:** *Introduction, "Here, we describe a novel methodology to co-amplify all four EGFR exons 18–21 along with KRAS exon 2 in a single multiplex PCR". It's more like a novel application of a well described methodology supported by several previous references. The novelty, albeit rather incremental, is in combining exons from two different genes, using previously described approach. Should be stated as such.*

   Author's response: As suggested by the reviewer, we have dropped the term "novel". Our modified text reads as follows:

   "….Here, we describe a methodology to co-amplify all four *EGFR* exons 18–21 along with *KRAS* exon 2 in a single multiplex PCR…."

5. **Referee's comments:** *The big appeal of the study is that it affords use of a minimal amount of FFPE sample. Please specify the amount of FFPE material used and yield of DNA to convey an idea of how little/much sample is needed to carry out this analysis.*

   Author's response: As mentioned in the methodology section subtitled, "Multiplex PCR of *KRAS* exon 2 and *EGFR* exons 18-21", multiplex PCR (50µl per reaction) was carried out in a single tube by using multiplex PCR kit (Qiagen) containing either 10 ng of genomic DNA from the NSCLC cell line or fresh frozen primary tumor, or 50 ng of genomic DNA from FFPE blocks. Furthermore, as mentioned under the methodology section subtitled, "Concatenation of exons and sequencing analysis", 2 µl of multiplex PCR product was used as template in a 50 µl PCR reaction for concatenation.

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   Author's response: As mentioned under the last paragraph of the discussion section, "….As the limitation of the CRE strategy is defined by the sensitivity and resolution of the sequencing methodology adopted…" – which in this study has been Sanger Sequencing-- as the sensitivity of Sanger Sequencing is well established from FFPE and mutated cell line, we humbly differ from the reviewer that admixture experiment would add additional information.

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   Author's response: *KRAS* codon 12 is mutated at a frequency of 25-50% in Caucasian population and 5-15% among East Asians. In a recent study we reported 18.6% *KRAS* codon 12 among Indian population (n=86). Given that *KRAS* codon 61 mutation exist at frequency < 1%; and, that none were found in our study in 86 patients, we decided to not include *KRAS* codon 61 mutation in this study to only present the proof of principle of the CRE methodology. However, we do agree with the reviewer about the significance of *KRAS* codon 61 mutation, and do hope to include it along with other known activating mutations in NSCLC.

We submit that based on literature, additional exons can be added to the current methodology, as at least up to 10 genomic spliced exons fragment of 2295 bp has been described in literature using similar methodology.

8. **Referee's comments:** *The concatenated PCR product may be amenable to Pyrosequencing to improve sensitivity of detection (particularly in case of low tumor content, low clonality of mutations as is expected in case of dynamically evolving tumors). This should be attempted/ discussed.*

   Author's response: We fully agree with the reviewer's insights that CRE can be utilized at high throughput mode to determine complete spectrum of *EGFR* and *KRAS* mutations using targeted next generation sequencing. Consistent with the reviewer's suggestion the last paragraph of the discussion section reads, "… the limitation of the CRE strategy is defined by the sensitivity and resolution of the sequencing methodology adopted, concatenated *EGFR* and *KRAS* PCR products from multiple individuals—each tagged with unique bar code sequence—can be pooled and deep-sequenced using a NGS platform. The CRE strategy described here can reduce the labor and cost of performing individual PCR for all exons for each patient and effectively circumvent the noise due to variation in normalization for equimolar pooling of exons within the same sample at a resolution of single base."

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   Author's response: As detailed in the manuscript, this proof of principle study introduces CRE as a methodology involving single multiplex-PCR followed by concatenation of the PCR product as one linear fragment for direct sequencing, as opposed to 5 rounds of PCR reaction followed by 10 rounds of sequencing reactions. A systematic comparative analysis is currently underway at our center using clinical cancer specimens for CRE compared to Sanger sequencing based methodology; SNaPShot PCR; Cobas system; Mass spec genotyping on a larger cohort sample, beyond the scope of this manuscript. Hence, we express our inability to include analysis from this ongoing study at this early on stage.

We sincerely thank the reviewer for the elaborate and detailed constructive review. Hope the reviewer will find the improved version of the manuscript acceptable for indexation.

Competing Interests: No competing interests were disclosed.