The detection of primary and secondary EGFR mutations using droplet digital PCR in patients with nonsmall cell lung cancer

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ABSTRACT

Background: We share our experience of using droplet digital polymerase chain reaction (DdPCR) in liquid biopsy specimens for detecting primary and secondary epidermal growth factor receptor (EGFR) mutations among patients with nonsmall-cell lung cancer who had tissue biopsy initially analyzed for del19, L858R and T790M. Materials and Methods: Three groups of patients were chosen: Group 1: patients positive for EGFR mutation (del 19 or L858R) by conventional tissue biopsy that were treatment naïve, Group 2: patients positive for EGFR mutation (del 19 or L858R) by conventional tissue biopsy with acquired resistance to tyrosine kinase inhibitor (TKI) therapy, documented by radiology, and Group 3: no known EGFR mutation detected on primary tissue biopsy and treatment naïve. Results: One hundred and thirty-three patients were included in the study. Group 1 had 40 cases, of which 21 (52.5%) and 19 (47.5%) were positive for del19 and L858R mutations, respectively, by tissue biopsy. DdPCR detected primary mutation in all but 5 cases. DdPCR additionally found four patients to have T790M mutation. Group 2 had 73 cases and DdPCR detected T790M mutation in 39 (53.4%) cases. Liquid biopsy also picked the original primary mutation in 56/73 cases. Secondary tissue biopsy for T790M mutation status was performed in 11 patients and while it detected mutation in 2 out of 11 cases, DdPCR additionally detected T790M mutation in 7 cases. Tissue biopsy additionally detected c-MET amplification in a patient who had T790M mutation on liquid biopsy. Group 3 had 20 patients and none were falsely positive for EGFR mutation on liquid biopsy. Overall, DdPCR had a Cohen's kappa of 0.82 (standard error 0.074, 95% CI 0.68–0.97) indicating "very good agreement" with conventional tissue biopsy. Conclusion: DdPCR demonstrated 87.5% sensitivity and 100% specificity in detecting primary EGFR mutations in patients who were treatment naïve with overall positive and negative predictive value of 100% and 80%, respectively. DdPCR demonstrated T790M mutation postprogression on TKI therapy in 53.4% patients.

KEY WORDS: Droplet digital polymerase chain reaction, epidermal growth factor receptor, liquid biopsy, nonsmall-cell lung carcinoma, polymerase chain reaction

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INTRODUCTION

Circulating tumor nucleic acid (ctDNA) is a source of tumor-derived genetic material. ctDNA is shed into the vasculature from tumor deposits as a result of apoptosis.\(^{[1-3]}\) The concept of cell-free nucleic acid was first introduced by Mandel in 1948. However, it was only in 1977 it came to be known that cell-free nucleic acid is increased in cancer patients.\(^{[4]}\) It took another 17 years to pick up the first KRAS mutation in plasma of pancreatic cancer patient employing polymerase chain reaction (PCR).\(^{[5]}\) Analyzing cell-free DNA or RNA (cfDNA/RNA) has certain advantages over tissue specimens and imaging such as repeated sampling is possible, tissue heterogeneity is addressed, no procedural complications, and no exposure to radiation.\(^{[6-10]}\)

cfDNA can be detected in plasma by various methods such as next-generation sequencing (NGS), amplification-refractory mutation system (ARMS) PCR, and digital platforms such as droplet digital PCR (DdPCR), each of which varies in sensitivity.\(^{[11]}\) It is reasonable to expect more sensitive platforms to have a better yield. DdPCR is one such machine, first time introduced in 1999, which has made accurate detection of scanty mutant fragments possible. It provides absolute gene quantification and does not require external calibrators making it possible to detect allele frequencies as low as 0.4%.\(^{[12]}\) Nonsmall-cell lung cancer (NSCLC) is at the forefront of utilizing these new age technologies to provide useful patient information based on which molecular-targeted treatment can be initiated or altered. The advent of targeted molecular therapy in the form of tyrosine kinase inhibitors (TKIs) has demonstrated a significant progression-free survival advantage in epidermal growth factor receptor (EGFR) mutant patients (primary mutation). During the course of first-generation TKI, the majority of tumor cells adapt or mutate to eventually become resistant to TKI therapy. The acquired resistance usually develops after 8–14 months of treatment.\(^{[13]}\) Nearly, 2 of 3 cases of progression with first-generation EGFR TKIs are related to the T790M mutation (secondary mutation).\(^{[14]}\) Other mechanisms of resistance development include amplification in c-MET and Her2 genes, mutations in BRAF and PIK3CA,\(^{[15-17]}\) epithelial-to-mesenchymal transition (EMT), and conversion to small-cell lung cancer. With the development of third-generation EGFR, TKIs which are effective against T790M mutation, an urgent need to identify the mutant clone is created. This has been conventionally done by repeating tissue biopsy (secondary biopsy).\(^{[18,19]}\)

Lung biopsy in the primary setting has a limited success rate with scarce tissue left for molecular testing in 20%–30% of the cases.\(^{[20]}\) The secondary biopsy is even trickier in already compromised and progressed Stage IV patients. Furthermore, heavily metastatic tumors may be heterogeneous with respect to acquired mutations and selecting a single site for biopsy has a chance of missing the resistant T790M mutation. Hence, the need for a noninvasive methodology which is representative of all tumor sites arises.

In this study, we share our experience of detecting primary and secondary EGFR mutations in liquid biopsy specimens using DdPCR among Indian patients with NSCLC.

MATERIALS AND METHODS

Study population

Ours is a regional cancer center based in northern India, providing tertiary care to referred patients. Following the institutional ethics committee approval (reference number 480/PA/AMH-14), the study was done for an arbitrary duration of 1 year from December 2016 to November 2017 in patients with metastatic NSCLC (Stage IV based on 8\(^{th}\) UICC TNM classification edition) having tissue biopsy at the time of initial diagnosis analyzed for EGFR exon 19 deletion (nucleotides 746_750/del 19) and L858R mutations, and whose blood samples could be obtained. We restricted analysis to three groups of patients for specific purpose as below:

- **Group 1:** Patients positive for EGFR mutation (either Del 19 or L858R) by conventional tissue biopsy who were treatment naive. This group was chosen to assess sensitivity of DdPCR as a diagnostic test upfront among patients with druggable mutation
- **Group 2:** Patients positive for EGFR mutation (either Del 19 or L858R) by conventional tissue biopsy and treated by EGFR-targeted TKI therapy with radiographic evidence of disease progression (acquired resistance). This group was chosen to assess performance of DdPCR in detecting secondary T790M mutation
- **Group 3:** No known EGFR mutation diagnosed on tissue biopsy (and hence not ideal candidate for TKI therapy) and treatment naive. This group was chosen to assess specificity of DdPCR in ruling out mutation status.

Sample collection and processing

All blood samples were tested by DdPCR-based plasma genotyping for detecting EGFR exon 19 deletion, L858R and T790M mutations. Samples from venous blood were collected in EDTA tubes and underwent centrifugation within 1 h for plasma preparation. Extraction of cfDNA was then performed using the QIAamp circulating nucleic acid kit (Qiagen) according to the manufacturer’s protocol. DNA was eluted in 100 uL of AVE buffer and stored at – 20°C until genotyping was performed. Genotyping of cfDNA was performed by DdPCR (BioRad) using primer/probes from BioRad. For this PCR-based technology, cfDNA is emulsified into approximately 20,000 droplets. PCR reaction takes place in the droplets subsequent to the addition of appropriate primer/probe. The results are read by a flow cytometer. The fluorescence signals are quantified to determine the number of copies of the mutant allele and the fractional ratio of mutant copies with respect to wild and mutant alleles. Tissue genotyping was performed for all patients.
on initial biopsy material using Therascreen® EGFR RGQ PCR kit from Qiagen.

**Statistical methods**
Continuous data are reported as median (range) and frequencies are reported as numbers (proportion). Count data for independent and paired groups were analyzed using Fisher’s exact F-test and McNemar’s test, respectively. Diagnostic utility for DdPCR was analyzed in terms of sensitivity, specificity, area under receiver operating characteristic area under curve (AUC), and positive and negative predictive value against tissue biopsy result as gold standard. Since none of the above tests provide a composite measure of “concordance” between DdPCR and tissue biopsy, Cohen’s kappa was additionally determined to assess “agreement” between both diagnostic modalities. Results were reported with 95% confidence interval (CI) and standard error wherever appropriate and all statistical analysis was performed using MedCalc Statistical Software version 15.8 (MedCalc Software bvba, Ostend, Belgium) assuming two-tailed alpha <0.05 as significant beforehand wherever appropriate.

**RESULTS**

One hundred and thirty-three patients formed the study population. Group 1, 2, and 3 had 40, 73, and 20 patients, respectively. Complete demographic, clinical, radiological, pathological, and molecular profile of the study population is described in Table 1.

**Group 1**

Twenty-one (52.5%) and 19 (47.5%) patients in Group 1 were found to have del 19 and L858R mutations, respectively, on tissue biopsy [Figure 1]. DdPCR successfully detected all but five cases with no specific predilection to miss any particular mutation subtype beyond chance (19/21 del 19 mutations picked, 16/19 L858R picked, \( P \) value 1). Furthermore, four patients had T790M mutation detected on DdPCR along with primary mutation which was not seen in the initial tissue biopsy. Among five cases who were not picked by DdPCR, one had disease limited to mediastinum while others had two or more metastatic sites beyond mediastinum. Thus, DdPCR had 87.5% sensitivity (95% CI 73.2%–95.8%) and 100% positive predictive value (95% CI 90%–100%) for detecting primary EGFR mutation in this specific subgroup.

**Group 2**

DdPCR detected secondary T790M mutation in 39/73 (53.4%) cases. DdPCR also detected the primary mutation in 56/73 (76.7%) cases. Among them, 35/56 (62.5%) were del 19 and 21/56 (37.5%) were L858R mutations. There was no statistically significant difference in relative observed proportions of del 19 and L858R mutations when compared to those observed in Group 1 \( (P = 0.40) \). Duration of secondary group testing from date of primary diagnosis ranged from 4 to 67 months. The results of secondary tissue biopsy to detect resistant T790M mutation were available for 11 patients, nine out of which primarily had EGFR del 19 and two had L858R mutation on initial tissue biopsy. Two of these secondary biopsies were performed from new lung lesions, five from new metastatic liver lesions, three from bony lesions, and one from supraclavicular lymph node. While secondary tissue biopsy detected T790M mutation in only 2 out of 11 cases, DdPCR detected the same in 7 cases, thus providing 45.5% superior yield with a trend toward statistical significance (McNemar’s test, \( P = 0.063 \)). DdPCR

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**Table 1: Demographics, clinical, radiological, pathological, and molecular details of entire study population \((n=133)\)**

| Age               | Range (years) | Median |
|-------------------|---------------|--------|
| Group 1           | 48–72         | 56.7   |
| Group 2           | 37–79         | 57     |
| Group 3           | 49–72         | 57.9   |

| Sex distribution | Males (%) | Females (%) |
|------------------|-----------|-------------|
| Group 1          | 25/40 (62.5) | 15/40 (37.5) |
| Group 2          | 31/73 (42.4) | 42/73 (57.5) |
| Group 3          | 10/20 (50)   | 10/20 (50)  |

| Smoking history | Nonsmokers | Smoker | History not available |
|-----------------|------------|--------|-----------------------|
| Males | Females |
| Group 1 | 18 | 9/12 | 3/12 | 10 |
| Group 2 | 13 | 22/27 | 5/27 | 33 |
| Group 3 | 15 | 3/3 | 0/3 | 2 |

| Metastasis | Limited to mediastinum | Extrathoracic metastasis | One site | More than one site |
|------------|------------------------|--------------------------|----------|-------------------|
| Group 1    | 14                     | 15                       | 11       |
| Group 2    | 12                     | 18                       | 43       |
| Group 3    | 4                      | 5                        | 1        |

| Histology | Adenocarcinoma | 102/133 |
|-----------|----------------|---------|
| NSCLC     | 30/133         | (not otherwise specified) |
| Adenosquamous | 1/133        | |

| Mutations on tissue biopsy | Del 19 | L858R | T790M |
|---------------------------|--------|-------|-------|
| Group 1                   | 21/40  | 19/40 | 0     |
| Group 2                   | 9/11   | 2/11  | 2/11  |
| (available for 11 patients only) |||
| Group 3                   | 0/20   | 0/20  | 0/20  |

| Mutations on liquid biopsy | Del 19 | L858R | T790M |
|----------------------------|--------|-------|-------|
| Group 1                    | 19/21  | 16/19 | 4/40  (3 with Del 19 and 1 with L858R) |
| Group 2 (primary mutation not picked up in 17 patients, \( n = 73 \)) | 35/56  | 21/56 | 39/73 |
| Group 3                    | 0/20   | 0/20  | 0/20  |

NSCLC: Nonsmall-cell lung cancer
also detected the primary mutation in all 11 cases. One case had both c-MET gene amplification on secondary tissue biopsy and T790M mutation on liquid biopsy [Table 2].

**Group 3**
All 20 cases in Group 3 were negative for known EGFR mutations on tissue biopsy and reassuringly none were detected as falsely positive for del 19, L858R, and T790M EGFR mutation on liquid biopsy, resulting in 100% specificity and 100% negative predictive value of a DdPCR test result in this group.

**Overall performance**
Thus, combining Groups 1 and 3 together (Group 2 was omitted as TKI treatment resistance is expected to be associated with suppressed sensitive EGFR clones), DdPCR had a Cohen’s kappa of 0.82 (standard error 0.074, 95% CI 0.68–0.97) indicating “very good agreement” with conventional tissue biopsy. DdPCR detected primary mutation correctly in 35/40 patients with overall sensitivity of 87.5% (95% CI 73.2%–95.8%), positive predictive value of 100.00% (95% CI 90%–100.00%), and AUC of 0.94 (standard error 0.027, 0.84–0.98, P < 0.0001). Further, none of the cases negative for EGFR mutations were falsely detected as positive in the study population resulting in an overall specificity of 100.00% (95% CI 83.16%–100.00%) and a negative predictive value of 80% (95% CI 59.3%–93.2%).

**DISCUSSION**
We studied the role of liquid biopsy in detecting primary and secondary mutations using DdPCR in NSCLC patients. This is the first prospective liquid biopsy data in NSCLC patients on a digital platform from India. We subdivided patients into three groups, each chosen with a purpose to be translated into useful clinical information. We could only come across one previous study with a design similar to ours; however, they did not include patients with absent EGFR mutation and hence could not comment on specificity of this test.[21]

Various platforms have been used to detect cell-free DNA. These have utilized scorpion-ARMS technology, real-time COBAS platform and PNA (PCR) clamping, DdPCR, beaming, and NGS.[11] Each platform varies in sensitivity. However, best results in terms of sensitivity and specificity have been historically observed with digital platforms.[22] Recently, modifications in NGS have shown improved performance;[23] however, it is expensive, time consuming, and yet performs almost similar to DdPCR in terms of sensitivity for detecting specific mutations.

DdPCR had an overall sensitivity and specificity of 87.5% and 100%, respectively, among treatment naïve patients. Our results concur with previous literature which reported a sensitivity of primary EGFR mutation detection between 77% and 87.5% and specificity between 96% and 100% on this platform.[21,24-26] Liquid biopsy is undoubtedly a good substitute in cases where tissue biopsy is not available. There are many situations in lung cancer patients where tumor tissue is inadequate for mutational analysis and performing repeat tissue biopsy in these Stage IV patients may be tricky. Further, liquid biopsy has the potential to pick up heterogeneous clones which may be missed in single site biopsy.[27-29]

DdPCR has picked relatively lower number of primary mutations in the Group 2 compared to the other groups. These patients were on TKI therapy which could possibly have suppressed the driver clone. Sacher et al. have reported sensitivity of liquid biopsy for primary mutation on the digital platform from 74% to 82% for various mutations in post-TKI progression cases (76.7% in our series).[21]

DdPCR detected T790M mutation in 4 out of 40 treatment naïve patients. This mutation was not picked up in the tissue biopsy which was performed using Therascreen®
EGFR RGQ PCR kit from Qiagen. This is owing to the fact that latter platform is not as sensitive as digital platform to detect these mutations. Previous meta-analysis of 3231 patients by Chen et al. has also shown that 12.9% of primary EGFR mutant patients also initially have T790M mutation. These cases were reported by various methods on both tissue and plasma with detection rate ranging from 0.32% to 78.95%.[36-40] When initially present, T790M clone is likely to overgrow after the driver mutation responds to TKI treatment. Many studies have reported a poorer survival in this category of patients.[31-35] Furthermore, these patients should be followed up to look for early resistance development.

DdPCR demonstrated T790M mutation postprogression on TKI therapy in 53.4% cases in our study. Previous studies have reported T790M positivity from 28% to 50% on various platforms with digital platform performing better than most real-time PCR-based platforms.[36-40]

Results of secondary tissue biopsy comparison with liquid biopsy were available in 11 patients only, all of which showed a primary mutation in both tissue and liquid biopsy. The drug against the resistant T790M clone has only been available in India since September 2017 and limited secondary biopsies were only performed under strong suspicion of small cell transformation or EMT.[16,18] T790M mutation was found in two tissue biopsy samples and seven liquid biopsy samples. Former two samples were also positive on liquid biopsy for T790M. AURA extension and AURA2 phase II studies using COBAS RT platform have reported overall predictive agreement of T790M reported in tumor tissue and plasma samples to be 65%,[41] Ahsan et al. have reported concordance of around 77% on DdPCR for T790M.[42] Although statistical significance was not reached for superior performance of DdPCR over tissue biopsy for detecting secondary mutations, our study is limited in numbers of congruent secondary tissue biopsies and thus is more likely to be a type 2 statistical error rather than true absence of difference in effect. In fact, one case showed MET amplification on tissue biopsy and T790M on liquid biopsy. Although all samples were not tested for the same, this particular case probably represented more than one mechanism of resistance.[42]

**CONCLUSION**

This study indicates the advantages of using an ultrasensitive technique of DdPCR in liquid biopsy to detect EGFR primary and secondary mutations in lung cancer patients. DdPCR demonstrated 87.5% sensitivity and 100% specificity in detecting primary EGFR mutations in patients that were treatment naïve with overall positive and negative predictive value of 100% and 80%, respectively. DdPCR demonstrated T790M mutation postprogression on TKI therapy in 53.4% patients.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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