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ORIGINAL ARTICLE

# Plasma next generation sequencing and droplet digital PCR-based detection of epidermal growth factor receptor (EGFR) mutations in patients with advanced lung cancer treated with subsequent-line osimertinib

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**Keywords**
Circulating tumor DNA; droplet digital PCR; next generation sequencing; plasma genotyping; resistance mechanisms.

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## Abstract

**Background:** Gene mutation analysis from plasma circulating tumor DNA (ctDNA) can provide timely information regarding the mechanism of resistance that could translate to personalised treatment. We compared concordance rate of next generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR) in the detection of the EGFR activating and T790M mutation from plasma ctDNA with diagnostic tissue biopsy-based assays. The second objective was to test whether putative osimertinib resistance associated mutations were detectable from plasma using NGS.

**Methods:** From January 2016 to December 2017, we prospectively collected plasma samples from patients prior to commencement of second- or third-line osimertinib therapy and upon disease progression, in a single tertiary hospital in South Western Sydney, Australia. Amplicon-based NGS and ddPCR assays were used to detect activating epidermal growth factor receptor (EGFR) and T790M mutations in 18 plasma samples from nine patients; all patients were required to have tissue biopsies with known EGFR status.

**Results:** High concordance of allelic fractions were seen in matched plasma NGS and ddPCR for activating EGFR mutations and T790M mutations ($R^2 = 0.92$, $P < 0.0001$). Using tissue biopsies as reference standard, sensitivity was 100% for NGS and 94% for ddPCR. Several possible osimertinib resistance associated mutations, including PIK3CA, BRAF and TP53 mutations, were detected by NGS in samples upon progression on osimertinib therapy.

**Conclusion:** ddPCR assays for EGFR mutations appear to be as sensitive and highly concordant as amplicon-based NGS. NGS has the ability to detect novel resistance mutations.

## Introduction

Mutational analysis of circulating tumor DNA (ctDNA) represents one of the major breakthroughs in thoracic oncology in recent years. ctDNA isolation from liquid biopsies allows identification of cancer-related mutations, which have significant impact on the management of patients, especially when tissue biopsy samples are not readily available. In lung cancer patients with acquired resistance to first generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), ctDNA

  

testing allows a rapid and noninvasive access to identify the presence of the resistance-associated acquired mutation T790M that predicts response to the third-generation EGFR-TKI, osimertinib.[1] Recent meta-analysis has shown that T790M can be detected in plasma ctDNA with high sensitivity and specificity.[2] Patients treated with osimertinib may subsequently develop further mechanisms of acquired resistance and have disease progression after a median treatment duration of 10 months.[3] Liquid biopsies could be used to monitor and guide treatment options based on the identification of mechanisms of acquired resistance.

Current available assays for analysis of plasma ctDNA are limited by cost, turnaround time and variable accuracy. The most widely used assay is droplet digital polymerase chain reaction (ddPCR) which is restricted to detection of known mutations and the limited capability of detecting only one mutation per assay. Next generation sequencing (NGS) of ctDNA is attractive due to its potential to more broadly detect multiple tumor specific genetic changes. Although whole genome NGS is still challenging for ctDNA, amplicon-based NGS targets specific disease related genetic hotspots[4] and is the basis for the tumor NGS assay that recently received approval by the US FDA (Oncomine Dx Target, ThermoFisher). Here, we compared ddPCR and amplicon-based NGS for detection and monitoring of driver and resistance mutations from ctDNA of advanced EGFR+ NSCLC patients. We propose that NGS will be useful for broader assessment of resistance mechanisms for patients who have progressed on osimertinib, while ddPCR assays have a role in monitoring disease response and progression.

## Methods

Nine patients with advanced EGFR+ T790M+ NSCLC and tissue biopsies available for evaluation were consented for plasma collection at Liverpool Hospital, Australia under approved Human Research ethics committee protocols (Project number 13/097, HREC/13/LPOOL/158). A total of 18 plasma samples were collected for analysis prior to commencement of osimertinib, and at the time of disease progression on osimertinib. Radiological imaging was performed to assess disease response to treatment at three and six months as per local treatment guidelines, with subsequent scans performed as per treating clinician's discretion. Plasma analyses were blinded to clinical data.

Statistical concordance of mutant allelic fraction (MAF) between plasma ddPCR and NGS was calculated using Kendall concordance coefficient. Sensitivity for plasma ddPCR and plasma NGS assays was calculated with reference to available tumor biopsy genotype.

### Plasma collection and ctDNA extraction

Peripheral blood samples were collected in 9 mL EDTA vacutainer tubes at the above time points. Plasma was processed within two hours and involved a first centrifugation step of 800 *g* for 10 minutes and a second spin of 13 000 rpm for 10 minutes. Plasma was stored at −80°C until ctDNA extraction using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturers' instructions. ctDNA was eluted in 20 μL per mL of input plasma and stored at −20°C until further analysis.

### Droplet digital PCR (ddPCR)

ddPCR was performed using Bio-Rad QX200 Droplet Digital system (BioRad Laboratories) for all samples at our centre with methods described previously.[5,6] Primers and probes were generated to detect EGFR-L858R, -S768I, -L861Q, -T790M and the corresponding wild type (WT) amplicons as well as the EGFR exon 19 deletion using the QX200 BioRad droplet digital PCR (ddPCR) suite (BioRad, Gladesville, Australia). The assays were optimised for annealing temperatures combined with Bio-Rad's recommended primer and probe concentrations and standard droplet digital PCR (ddPCR) protocol. Detected mutant and wild type copy numbers were normalised based on the volumes of plasma used for DNA extraction, elution and the input into the ddPCR reaction (DNA copies/mL plasma) based on the following formula:

$$\frac{Events \times 20}{Input\ ctDNA\ volume\ (\mu L)} \times \frac{Elution\ volume\ (\mu L)}{Plasma\ volume\ (\mu L)}$$

The ddPCR threshold for positive mutant detection was set at two or more positive droplets (≥10 mutant copies/mL of plasma) to ensure specificity. The sensitivity of our ddPCR assay allowed for the detection of a mutant allele fraction of 0.1% or more (i.e., one mutant molecule in a background of 1000 wild type molecules).

### Plasma next generation sequencing (NGS)

The DNA panel of Oncomine Lung cfTNA Research Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for this study targeting 11 genes with 174 hotspot SNVs and indels, including ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1, and TP53. ctDNA were quantitated using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Tag Sequencing technology was employed to amplify regions of interest. Sequencing libraries were prepared with Oncomine Lung cfTNA Research Assay kit (Thermo Fisher Scientific) following the manufacture's protocol using 20 ng of DNA to achieve 0.1%

 

limit of detection (LOD); then libraries were quantified by qPCR with Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific). Libraries with concentration ranging from 154 to 2364 pM were obtained and normalized to 25 pM. Five to six normalized libraries were pooled together, templated on Ion Chef System, loaded onto Ion 520 Chips, then sequenced on Ion S5 XL sequencer. Reads were mapped back to the designed target regions supplied by the manufacturer automatically by using Oncology-Liquid Biopsy workflow on Torrent Server with Torrent Suite software version 5.6 (Thermo Fisher Scientific). Variants were called using default set parameters of Oncomine Lung Liquid Biopsy - w1.3 - DNA - Single Sample 5.6 workflow on local Ion Reporter Server (Thermo Fisher Scientific) at IR5.6.

## Results

### Patient and disease characteristics

A total of 18 paired plasma samples from nine patients were collected before commencement on osimertinib and post-osimertinib resistance. All patients had been previously diagnosed with EGFR+ advanced NSCLC with tissue biopsy testing being positive for common and uncommon EGFR mutations. Tissue histopathology and EGFR mutation testing were performed as part of the routine diagnostic testing by the anatomical pathology laboratory at Liverpool Hospital, NSW, Australia. The therascreen EGFR RGQ PCR kit (Qiagen, Hilden, Germany) was used with targeted ARMS (amplification refractory mutation system) technology to detect mutations in exon 18–21 of the EGFR gene. All patients had lung adenocarcinoma histological subtype. All patients had a minimum of two sites of extracranial metastatic disease (range 2–5 sites). Other patient characteristics are shown in Table 1.

All patients were treated with first generation EGFR-TKI gefitinib or erlotinib with a median duration on treatment of 10.8 months (range 5.4–34.2 months). Upon radiological disease progression, 4/9 (44%) patients had repeat tissue biopsy with none showing histological transformation to small cell lung carcinoma. All patients had an EGFR-T790M mutation detected on tissue or plasma biopsy and were treated with osimertinib at a standard daily dose of 80 mg. Median duration of treatment with osimertinib was 7.3 months (range 2–19 months). At the first radiological assessment of response to treatment, 5/9 (56%) patients had a partial response with 4/9 (44%) showing radiological evidence of disease progression. 5/9 (56%) patients received chemotherapy post-osimertinib and 3/9 (33%) had best supportive care only. One patient was continued on osimertinib upon disease progression with stable disease on subsequent scans. At the time of data cutoff, 77% (7/9) patients had died. Median PFS was seven months (range 1–15 months) and median OS was 12 months (range 2–22 months). Figure 1 summarises the duration of subsequent treatment and survival for each patient.

### High concordance and sensitivity in detecting known EGFR mutations

We observed a correlation between quantification of total cell-free DNA (cfDNA) quantity (measured by Qubit) and the detected concentration of WT cfDNA for EGFR ($R^2$ = 0.81, $P < 0.0001$, Fig 2a). Quantitative concordance of allelic frequency (AF) between NGS and ddPCR was high for both EGFR activating and T790M mutations ($R^2$ = 0.92, $P < 0.001$, Fig 2b). Pre-existing diagnostic activating EGFR mutation in tissue biopsy was used as a reference standard to determine sensitivities of plasma NGS and ddPCR across the 18 plasma samples. Sensitivity for

**Table 1** Patient characteristics and detection of resistance mutations post disease progression on osimertinib

| ID | Gender | Smoking status | Duration on osimertinib (months) | Ethnicity | Tissue mutation | Number of sites of metastatic disease | Driver at resistance (allelic frequency) | Post-osimertinib resistance mutations (allelic frequency) |
|---|---|---|---|---|---|---|---|---|
| 1 | M | Ex-smoker | 3 | Asian | L858R | 3 | L858R (3.2%) | TP53 G245D (0.14%) |
| 2 | F | Ex-smoker | 6 | Asian | L858R | 5 | L858R (2.7%) | None detected |
| 3 | M | Smoker | 8 | Caucasian | L858R | 5 | L858R (82.7%) | TP53 R273H (24%) |
| 4 | M | Non-smoker | 5 | Caucasian | L858R | 4 | L858R (31.8%) | PIK3CA H1047R (24.4%) |
| 5 | F | Non-smoker | 11 | Asian | L858R | 2 | L858R (0.25%) | TP53 G154T (0.15%) |
| 6 | F | Non-smoker | 14 | Asian | Del19 | 3 | Del19 (4.13%) | TP53 C277Y (0.09%) |
| 7 | F | Non-smoker | 4 | Asian | Del19 | 2 | PIK3CA E542K (1.1%) | BRAF V600E (0.08%) |
| 8 | F | Ex-smoker | 1 | Caucasian | S768I/G719S | 4 | G719S (3.2%) | None detected |
| 9 | M | Smoker | 2 | Asian | L861Q | 4 | L861Q (5.8%) | None detected |

F, female; M, male.

  

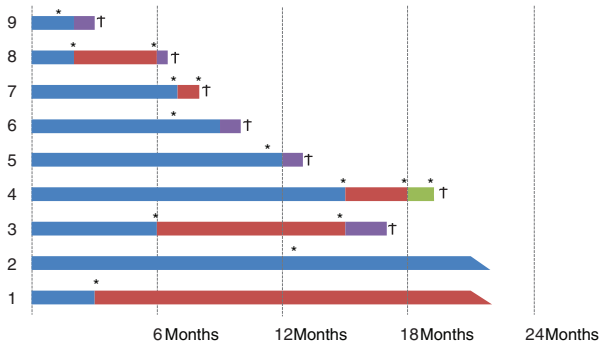


**Figure 1** Treatment duration and survival outcome of patients. Swimmer plot indicating treatment types and duration that patients received and survival outcome. All patients were started on osimertinib after disease progression with first-generation EGFR TKI or chemotherapy. (■) Osimertinib, (■) Chemotherapy (carboplatin/gemcitabine), (■) Nivolumab, (■) BSC, (✻) Disease progression, (†) Death and (▱) Ongoing therapy.

detection of the driver EGFR mutation was 100% (18/18) for NGS and 94% (17/18) for ddPCR.

### Detection of resistance mutations to osimertinib with plasma NGS

We investigated resistance mechanisms in nine osimertinib-treated patients with detectable EGFR driver mutations with plasma NGS on paired plasma samples (pre- and post-treatment resistance with osimertinib, Table 1). Four patients (44%) developed new TP53 mutations (G245D, C277Y, R273H, G154T). Two patients (22%) developed PIK3CA mutations (H1047R, E542K). We also detected the BRAF V600E mutation in the plasma of one patient at disease progression. Three patients (33%) had detectable T790M in the plasma at disease progression.

Two patients had uncommon EGFR mutations (S768I and L861Q) but there is little data currently to predict whether these patients will respond to third generation EGFR-TKIs. In our study, both these patients had disease progression within the first three months of treatment with osimertinib. Interestingly, no new mutations were identified with the lung panel NGS at disease progression in the two patients with these mutations, suggesting that these uncommon EGFR mutations may predict for primary resistance to osimertinib.

## Discussion

Plasma amplicon-based NGS is a relatively new technology, while ddPCR is now firmly established for ctDNA based biomarker detection. Our study demonstrated high sensitivity and quantitative concordance of amplicon-based plasma NGS when compared with ddPCR to detect EGFR-activating and resistance mutations.

Both methods are attractive for specific settings using ctDNA. Compared to NGS, ddPCR is proposed to have higher sensitivity down to 0.001%.[7] Further, it has an easier set-up process, faster turnaround time, and does not require complex informatics support for analysis. However, only known genetic changes can be detected, and the potential for multiplex detection of several biomarkers is limited. In contrast, NGS may identify any genetic changes in the entire target regions screened, including novel

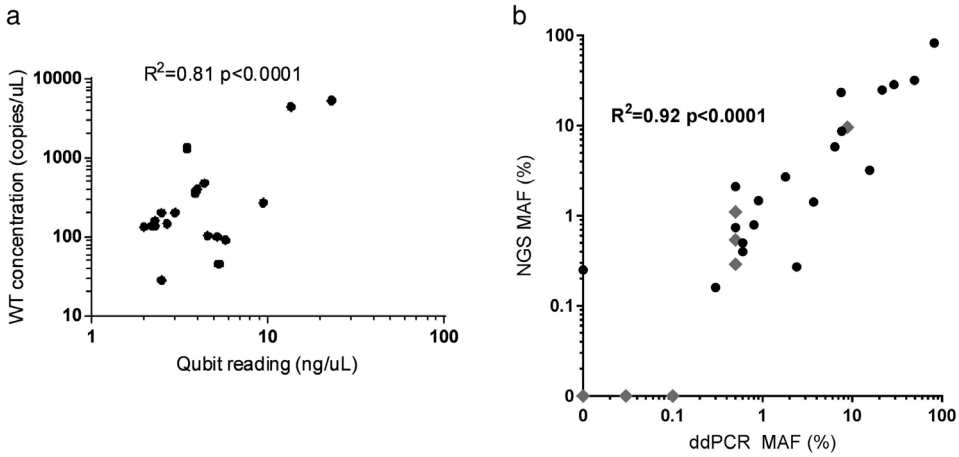


**Figure 2** (**a**) High concordance between Qubit reading and WT allele concentration ($R^2$ = 0.81). WT, wild type. (**b**) Concordance EGFR mutation detection ddPCR versus NGS. A total of 18 samples from nine patients were analysed with both ddPCR and NGS to assess EGFR mutations. There was high quantitative concordance ($R^2$ = 0.92) of the mutant alleic frequency (MAF) across 36 EGFR variants (activating: L858R, exon 19 deletion, L861Q, S768I and T790M resistance mutation) from 18 samples detected both with plasma NGS and plasma ddPCR. NGS, next generation sequencing; ddPCR, droplet digital polymerase chain reaction; MAF, mutant allelic frequency. (●) EGFR activating mutations, and (♦) EGFR T790M.

 

mutations. Testing of many genetic regions simultaneously is common with NGS. However, NGS is more time-consuming, more costly, and requires more complex informatics support for data analysis. A recent study has shown that amplicon-based NGS technology could also be used to detect chromosomal rearrangement and fusion genes in ctDNA from NSCLC patients with high sensitivity and specificity.[8]

Our study has added to the current knowledge on the potential mechanisms of acquired resistance to osimertinib. In our patient cohort, we detected the previously reported, and likely resistance-associated changes, BRAF V600E and PIK3CA using plasma NGS[9]. We have not detected other secondary EGFR mutations such as C797 and L792 or C-MET amplification which were reported to be present in 26% and 14% of cases respectively[10] likely due to the sample size. There were four cases of acquired TP53 mutations at the time of osimertinib resistance, with patient 3 reaching a very high allelic frequency of 24% (Table 1). TP53 mutation is commonly detected in human cancers and its product is a multifunctional protein that regulates several physiological processes including cell cycle checkpoints, apoptosis and DNA repair.[11] Currently, there is limited knowledge regarding the role of mutant p53 in osimertinib resistance or the mechanism involved. A previous study in triple negative breast cancer suggested that mutant p53 may be linked to endosomal recycling of EGFR and integrin to the membrane, leading to increased proinvasive abilities of cancer cells.[12] Functional studies are necessary to clarify if TP53 mutations detected here indeed alter EGFR levels, or whether its proapoptotic role is crucial for osimertinib induced cell death with the implication that mutations may lead to osimertinib resistance in NSCLC.

It was interesting that no new potential resistance mutations were identified for two patients with EGFR S768I and L861Q activating mutations using the lung panel NGS at the time of disease progression, and that both patients showed resistance to osimertinib within three months of therapy. Pooled data analysis had shown that these mutations predict response to the second generation EGFR-TKI afatinib,[13] but whether these mutations predict sensitivity to osimertinib is currently unclear. If these mutations are the cause of the primary resistance to osimertinib, it would be important to clarify this by functional in vitro assays to guide treatment decision for patients with these uncommon EGFR mutations in the future.

The main limitation of this exploratory study is the small number of patients included. Larger studies will be needed to validate the findings from this study. Nonetheless despite the small number, this study adds to the evidence that NGS and ddPCR have high concordance and accuracy in detecting EGFR sensitizing and resistance mutations in lung cancer patients. This study also showed that new mutations could be detected in the plasma of almost all patients who developed acquired resistance to osimertinib. We acknowledge that the patients in this study were heterogenous and it is possible that the patients' clinical characteristics (e.g., ethnicity, smoking status and treatment prior to osimertinib) may affect treatment outcome and pattern of acquired resistance to osimertinib. A previous meta-analysis showed that patients with exon 19 deletions, never smokers and female have better treatment outcomes with EGFR-TKI.[14] There is also preliminary data which showed that the pattern of acquired resistance is different when osimertinib is used in first-line setting[15] compared with second-line setting or beyond. Subgroup analyses (e.g., Asian vs. Caucasian; smokers vs. non-smokers) could not be performed in this small study and should be investigated in a larger study to investigate the difference in pattern of acquired resistance to osimertinib based on different clinical characteristics. Nonetheless this heterogeneous group of patients represents the real-world demographic of patients with EGFR-mutant NSCLC in a large tertiary hospital in South Western Sydney which contains an ethnically diverse population.

This small study has also highlighted one of the most important issues of whether commercially available NGS is ready to be integrated into routine clinical practice. One of the main advantages of characterisation of the resistance mutations is the potential this has to open up new treatment options including clinical participation for patients when the current treatment fails. We acknowledge that drug access varies in differing parts of the world; however, there are targeted treatments currently available in clinical practice for some of the mutations identified in our study including BRAF V600E. Conversely, for the four patients with acquired TP53 mutations, whilst there are no targeted therapeutics currently available, the knowledge of the presence of this abnormality may facilitate potential clinical trial participation. Clinical treatment paradigms will continue to evolve with more complete understanding of p53 biology and continued work as to how to optimally target aberrant p53 function. Further, knowledge of TP53 mutation status may help to risk stratify patients in the future and may help for treatment selection similar to the story of chronic lymphocytic leukemia with TP53 abberations.[16] Longer term follow-up results from large prospective studies which include molecular profiling with NGS for treatment selection will guide clinicians in determining the clinical utility of NGS in the management of solid tumors.

## Conclusion

While the amplicon-based plasma NGS assay provided coverage of the 11 major targetable gene alterations, specific alterations were confirmed by ddPCR results in almost

  

all cases, suggesting similar performance and accuracy of both these technologies. With the increase in accessibility of both technologies, we suggest that they may have complementary roles in diagnosis and treatment monitoring in metastatic cancer, depending on clinical questions at hand, cost, accessibility with appropriate bioinformatics support, and whether clinically-relevant genetic alterations are known.

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