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Detection of low-level *EGFR* c.2369 C>T (p.Thr790Met) resistance mutation in pre-treatment non-small cell lung carcinomas harbouring activating *EGFR* mutations and correlation with clinical outcomes

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Linda Ye: study design, data collection, data interpretation, writing the manuscript.

Nima Mesbah Ardakani: study design, molecular analysis, data collection, data interpretation, writing the manuscript.

Carla Thomas: molecular analysis, data collection, writing the manuscript.

Katrina Spilsbury: data analysis, writing the manuscript.

Connall Leslie: study design, writing the manuscript.

Benhur Amanuel: study design, molecular analysis, data interpretation, writing the manuscript.

Michael Millward: study design, data interpretation, writing the manuscript.

Abstract

Background: Increasing evidence points to the presence of low-level de novo T790M mutations in patients with non-small cell lung carcinoma (NSCLC) harbouring activating *EGFR* mutations. We utilised digital PCR (dPCR), a highly sensitive gene mutation detection method, to detect pre-treatment T790M mutations in NSCLC tumour samples and correlated the T790M status with clinical features and patient outcomes.

Materials and Methods: DNA extracted from pre-treatment NSCLC tumour tissue with known activating *EGFR* mutations, diagnosed between October 2010 and May 2017 at PathWest laboratory, was used to perform targeted dPCR for quantitative detection of T790M mutations.

Results: T790M was detected in 42 of 109 pre-treatment samples (38.5%). Median variant allele frequency was 0.14% (range 0.02% to 28.5%). Overall response rate to first generation EGFR tyrosine kinase inhibitors (TKI) was 67% regardless of T790M status. The median progression free survival was 10.7 (IQR 5.6 – 19.9) versus 6.7 (IQR 3.5 – 20.8) months in T790M negative and positive patients respectively. T790M positivity correlated with increased rate of early disease progression. It also correlated with increased mortality (HR 3.1 95%CI 1.2-8.1, p=0.022) in patients who did not respond to TKI treatment.

Conclusion: We detected a significant rate of low-level pre-treatment T790M mutations in NSCLC using highly sensitive dPCR. Low-level pre-treatment T790M did not impact treatment response rate or overall survival, but was associated with increased rate of early progression on TKI therapy.

Introduction

Major advances have been made in the identification of molecular biomarkers and targets for personalised treatment strategies for lung cancer patients in recent years. Activation of the epidermal growth factor receptor (EGFR) signalling pathway promotes neoplastic cell proliferation, invasion, angiogenesis and resistance to apoptosis. Activating *EGFR* mutations, most commonly exon 19 deletions and pL858R point mutation in exon 21, confer sensitivity to EGFR tyrosine kinase inhibitors (TKI) and are important therapeutic targets in non-small cell lung carcinoma (NSCLC). More recently, a secondary missense *EGFR* c.2369 C>T, (p.Thr790Met) mutation (T790M) was identified as the major cause of acquired resistance to first generation EGFR TKIs such as erlotinib and gefitinib ¹⁻⁴. This led to the development of third generation EGFR TKIs such as osimertinib that can effectively target the common *EGFR* activating mutations as well as T790M via irreversible inhibition ⁵. Osimertinib demonstrated improved response, survival and quality of life advantages over platinum and pemetrexed chemotherapy after progression first line EGFR TKIs and was rapidly adopted as the standard of care for T790M mediated EGFR TKI resistance ⁶. More recently, the FLAURA trial reported an overall survival benefit for osimertinib compared to erlotinib or gefitinib in the first line setting, supporting the use of osimertinib in treatment naïve patients ⁷.

There is emerging evidence supporting the presence of low-level T790M mutations in pre-treatment tumours in patients with NSCLC with subsequent clonal expansion following exposure to first generation EGFR TKIs ^{8,9}. The prevalence of pre-treatment T790M mutations in patients with *EGFR* mutant non-small cell lung carcinoma has not been clearly defined and detection rates ranging from 0% to 100% have been reported, depending on the detection method used and its analytical sensitivity ^{4,8-17}. Digital PCR (dPCR) is a highly sensitive quantitative mutation detection method that identifies variants based on the compartmentalisation and amplification of a single DNA molecule ¹⁶. Digital PCR has been applied to analyse somatic mutations in various cancer types and it has been shown to detect T790M mutations with higher sensitivity than other PCR based methods such as conventional DNA sequencing and the Amplification Refractory Mutation System (ARMS) ^{16,18}. The clinicopathological characteristics of tumours with pre-treatment T790M are unclear, as are the potential clinical implications. In this retrospective study we analysed and quantified T790M mutations in pre-treatment NSCLC tumour specimens with known activating *EGFR* mutations using dPCR and correlated the findings with

clinicopathological characteristics, response to EGFR TKI therapy, progression free survival and overall survival.

Materials and Methods

Patients and Samples Selection

Pathology records at PathWest laboratory were reviewed using AP system (version 8) and 150 patients with histologically confirmed NSCLC known to harbor activating *EGFR* mutations, diagnosed between October 2010 and May 2017, were identified. All patients had pre-treatment tissue samples that have been previously assessed for T790M mutations by either Cobas 4800 *EGFR* assay (Roche Molecular Systems Inc., Australia) or Sanger Sequencing or both assays. Sufficient DNA for dPCR testing was either available or could be re-extracted from tissue samples in only 109 patients. Clinical and demographic data were retrieved from medical records including age at diagnosis, sex, race, TNM stage (7th AJCC TNM Staging System), smoking history (either never smoked or current/ex-smoker), anti-cancer treatment history, response to EGFR TKI, duration of treatment and survival outcomes censored at 8/11/2018.

Progression free survival was defined as the time from the start of *EGFR* TKI treatment until the date of radiological or clinical disease progression determined by the treating physician. Overall survival was defined as the time from TKI treatment until death from any cause.

The study was approved by the Sir Charles Gairdner Hospital Executive Committee and Quality Improvement Committee (Approval number 16361).

Genomic DNA Extraction

Genomic DNA was extracted from formalin fixed paraffin embedded (FFPE) tissue using the Qiagen QIAamp DNA Extraction kit according to manufactures instructions (Qiagen, Australia). Extracted DNA was quantified by spectrophotometry using a Nanodrop ND-2000 (Nanodrop, USA).

Digital PCR (dPCR)

Digital PCR was carried out using the [™] QuantStudio® 3D Digital PCR System (Life Technologies, VIC, Australia) according to manufactures protocol. Samples were loaded onto the chips using the

QuantStudio® 3D Digital PCR Chip Loader in a mixture consisting of 7.5 µL of 2x Quantstudio® 3D digital PCR master mix, 0.8 µL of 20x EGFR_6240 TaqMan Probe (Thermo Fisher Scientific, VIC, Australia) and 6.7 µL of 50ng of diluted DNA. The chips were sealed and loaded onto a ProFlex™ 2x Flat PCR System with the following program: 96°C for 10 min, followed by 40 cycles of 60°C for 2 min and 98°C for 30 s, with a final 2 min incubation at 60°C. After cycling, the end-point fluorescence of the partitions on the chips was measured by transferring the chips to the measurement unit (application version 1.1.3, algorithm version 0.13) and analyzed with QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific).

Droplet Digital PCR (ddPCR)

The Bio-Rad QX200 ddPCR system was used and all reactions were prepared using the manufacturer's standard protocol (Bio-Rad, NSW, Australia). Each reaction contained 10 µL of 2x ddPCR Supermix (no dUTP) for Probes (Bio-Rad), 2 µL of 20x EGFR_6240 TaqMan Probe (Thermo Fisher Scientific) and 8 µL of 50ng of diluted DNA as template. Droplets were generated using the QX200 droplet generator (Bio-Rad) according to the manufacturer's protocols. The PCR cycling conditions were 96°C for 10 min, followed by 40 cycles of 60°C for 2 min and 98°C for 30 s, with a final 2 min incubation at 60°C. Droplets were read in the QX200 droplet reader, and analysed using the QuantaSoft Analysis Pro software, version 1.0.596 (Bio-Rad).

Establishing the Threshold for True Positive Results

The sensitivity of a mutation detection assay is defined by a threshold for making positive calls about mutational status on individual samples with a known level of confidence. For both dPCR and droplet dPCR, the baseline level of false-positive counts determines what concentration of true positives can be detected with statistical confidence. The limit of blank (LoB) is the highest amount of analyte (in this case T790M false positives) expected to be found in negative control samples and it is the primary characteristic of an assay that determines the limit of detection (LoD) threshold. The LoD is the lowest result reliably distinguished from the LoB and at which detection is feasible. The LoB and LoD were determined based on published studies by Armbruster and Pry (2008)¹⁹.

Briefly LoB was determined by calculating the mean and the standard deviation (SD) of FAM positives in multiple repeat assays using 50 ng of human male genomic normal reference DNA (Promega, Cat #

G1471) in the *EGFR* T790M assay. The LoB is then calculated using the following equation: $\text{LoB} = \text{mean}(\text{blank}) + (1.645 \times (\text{SD}_{\text{blank}}))$. Supplementary tables 1 & 3 contain the data from 15 repeats of a wild-type only DNA that were used to calculate the LoB.

The LoD for the assay was determined using the measured LoB and SD of five replicates of a sample known to contain a T790M variant at a low VAF. The LoD is then calculated according to the following equation: $\text{LoD} = \text{LoB} + [1.645 \times (\text{SD}_{\text{low VAF sample}})]$. Supplementary tables 2 & 4 contain the summarized data from 5 repeat assays of a FFPE sample with a low T790M VAF that was used to calculate the LoD.

The LoD was used to determine the criteria to call a sample positive for the *EGFR* T790M assay. For dPCR, a sample was called positive if it had a $\text{VAF} > 0.07\%$ or > 0.18 copies/ μL FAM, in addition to a minimum of 2 FAM events. If these criteria were not met, samples underwent repeat testing by dPCR +/- droplet dPCR. For droplet dPCR, a sample was called positive if it had a $\text{VAF} > 0.04\%$ or > 0.26 copies/ μL FAM, in addition to a minimum of 2 FAM events.

The above criteria were required to be met on repeat dPCR testing or droplet dPCR testing to be considered a true positive result. Samples that did not meet the criteria for positivity were categorised as *EGFR* T790M negative.

Statistical Analysis

Equality of proportions were assessed with chi-squared tests, mean with t-tests and medians using the Wilcoxon rank sum test. For survival outcomes, equality of expected versus observed events was assessed using log rank tests. Survivorship functions were plotted using the Kaplan-Meier method. Multivariable flexible parametric models using the restricted cubic spline method of Royston-Parmar were used to assess how the hazard rates of progression free survival and overall survival varied over time by T790M status²⁰. Time dependent effects (non-proportional hazards) were assessed using Schoenfeld residuals after a proportional hazards regression model. Potentially explanatory or confounding variables also tested for inclusion in the models were age, sex, smoking history, race, comorbidity, *EGFR* genotype, number of lines of palliative therapy, time between diagnosis and first line TKI treatment, best response to TKI treatment and year of diagnosis. Plausible interaction terms

of explanatory variables with T790M status and outcome were also assessed. Stata Statistical Software: Release 15 (Stata Corp, College Station, TX) was used.

Results

A total of 109 patients with known EGFR mutant NSCLC were included in this study. All patients had non-squamous cell carcinomas apart from a 65-year-old Caucasian female non-smoker who was diagnosed with squamous cell carcinoma on cytology. Pre-treatment tissue specimens used for analysis were from cytology samples (57%), core biopsies (37%) and tumor resection specimens (6%). There were 51 exon 21 mutations (47%) including 49 c.2573T>G (p.Leu858Arg) mutations (L858R) (45%) and 2 Exon 21 c.2582T>A (p.Leu861Gln) mutations, 46 exon 19 deletions (42%), 11 exon 18 mutations (10%) and five exon 20 mutations (5%) including one being T790M (Table S5 Supplementary Appendix). Nine tumours had two synchronous *EGFR* mutations

Pre-treatment tumour T790M mutations were detected by dPCR in 42 patients (38.5%) with allele frequencies as low as 0.02% (Table 1). dPCR results were confirmed and duplicated by droplet digital PCR in all samples with adequate DNA for repeat testing. The median mutant allele frequency was 0.14% and ranged from 0.02% to 28.5%. Of the 42 pre-treatment T790M positive cases, 14 (33.3%) had a mutant allele frequency of <0.1% and 28 (66.7%) had a mutant allele frequency of ≥0.1%. Only one tumour sample had pre-treatment T790M detectable by Sanger Sequencing in which the mutant allele frequency was identified as 28.5% by dPCR. This patient received first line gefitinib without any response and treatment was ceased after five weeks.

Patient Characteristics

Complete clinical data was available for 91 patients, including nine patients with stage I disease (9.9%), three patients with stage II disease (3.3%), one patient with stage III disease (1.1%) and 78 patients with stage IV disease (85.7%).

The subset of patients with stage IV disease who received first line first generation EGFR TKI therapy with either erlotinib or gefitinib (n=64) were investigated in more detail. Patient characteristics are described in Table 2. The distribution of pre-treatment T790M status did not differ by patient age, sex,

race, smoking status or common EGFR mutation genotypes. Pre-treatment T790M was detected in 23 cases (35.9%), including 10/24 (41.7%), 10/33 (30.3%) and 3/8 (37.5%) of tumours with activating EGFR exon 21 L858R mutation, exon 19 deletions and exon 18 mutations respectively.

Clinical Outcomes in Stage IV patients treated with first line Erlotinib/Gefitinib

Two-thirds (n=43, 67%) of the patients with stage IV disease treated with erlotinib or gefitinib had a partial or complete response to treatment. There was no difference in the proportion of responders to erlotinib or gefitinib by pre-treatment T790M status (p=0.598). The overall median progression free survival for the stage IV patients was 8.8 months (IQR 4.7-19.9). There was no significant difference in overall progression free survival by pretreatment T790M status (log rank test p=0.897) or T790M allele frequency dichotomized as <0.1 or ≥0.1% (p=0.515)

However, the pattern of the Kaplan Meier curve suggested that the rate of disease progression by T790M status varied over time, with a faster rate of disease progression in T790M positive patients during the first few months of treatment (Figure 1A and 1B). The variation in the rate of progression free survival by T790M status was further investigated as an exploratory analysis assuming non-proportional hazards over time. Statistical significance for time-dependent effects was confirmed (p=0.022) and is presented graphically in Figure S1 in the Supplementary Appendix. An increased rate of disease progression in T790M positive patients was evident up to six months post TKI treatment (HR 2.0; 95%CI 1.1-3.8) (Table S6 Supplementary Appendix). By six months, there was no significant difference in progression free survival based on pre-treatment T790M status. There was no association between age, sex, race, smoking history or other EGFR mutations and progression free survival in this cohort.

The median overall survival in stage IV patients treated with first line TKI was 2.0 years (IQR 0.9 – 4.2 years). The median overall survival for patients without T790M was 2.5 years compared with 1.8 years for those with T790M (Figure 2). There appears to be a trend of better overall survival in T790M negative tumours however this difference did not reach statistical significance (HR 0.91; 95%CI 0.50-1.64; p=0.750)

However, it was evident that the strength of association between pre-treatment T790M status and survival differed for patients who showed no response to first line TKI compared to those who did show

a response to treatment. Amongst the group of patients who did not respond to TKI treatment (n=21), those who had pre-treatment T790M died three times faster (HR 3.1 95%CI 1.2-8.1, p=0.022) than those without pre-treatment T790M. Whereas, there was no difference in survival based on T790M status in the responders to TKI treatment (HR 0.86; 95%CI 0.4-1.8; p=0.693). Increasing time between diagnosis and TKI treatment was independently correlated with improved survival. We did not find any association between survival and age, comorbidity, sex, smoking history, race, or EGFR genotype.

Clinical Outcomes in Patients Treated with Osimertinib

Two patients received first line osimertinib. In one patient pre-treatment T790M mutation was identified at a low allele frequency of 0.2% and the other patient was pretreatment T790M negative. The first patient received osimertinib for 18 months before disease progression and the second patient continued to have ongoing response at the time of census after 33 months of treatment.

There were 12 patients who received second line osimertinib after disease progression on first generation EGFR TKI. All of these patients had T790M detected in tumor or blood prior to commencing osimertinib via Cobas or Sanger sequencing or digital PCR. Seven patients derived a partial response (58.3%). The median progression free survival on second line osimertinib was 15.4 months in all patients who received such treatment; however, the median progression free survival 9.7 (IQR 4.6 – 15.4) months in those without pre-treatment T790M.

Discussion

In this study we demonstrated that a significant subset of patients with NSCLC and activating *EGFR* mutations also harbor low-level T790M (average 0.14% allele frequency) mutation in pre-treatment tumor samples and substantiated dPCR as a feasible and sensitive detection method for such low-level mutations. The prevalence of pre-treatment T790M mutations has been variable and a wide range has been previously reported in the literature. Studies utilizing conventional DNA sequencing methods have reported a very low prevalence of 0 – 3%^{2, 4, 8, 9, 13, 21-23}. However, results generated through more sensitive assays indicate a significantly higher prevalence with detection rates up to 38% observed with ARMS^{14, 24}. Multiple other methods have also been applied for detection of pre-treatment T790M

mutations, including peptide-nucleic acid-clamping PCR^{10, 23}, Colony Hybridization assay¹¹, matrix-assisted laser desorption ionization-time of flight mass spectrometry^{9, 17, 25}, mutant enriched PCR⁸ and TaqMan Mutation Detection Assay^{10, 15, 26, 27} with reported detection rates ranging from 2% to 78%. Although these methods have higher analytical sensitivity, most have not been commercialized for routine clinical practice.

dPCR is a sensitive and practical method that has been widely adopted for clinical practice for the detection of clinically important somatic variants including *EGFR* mutations. We detected T790M mutations in 38.5% of pre-treatment specimens with allele frequency as low as 0.02%, similar to results reported by Tatematsu et al²⁷. Two other Japanese studies have reported detection rates of 79.9%¹⁶ and 100% using dPCR¹² while another group has reported a detection rate of 28.6%²⁸.

The observed heterogeneity in the incidence of pre-treatment T790M mutations is mainly due to the variation in detection methods and their analytical sensitivity used in different studies; however, other factors such as differences in study population, the quality of tumor tissue and extracted genomic material as well as the extent of quality control to detect false positive/negative results may have been contributory. Highly sensitive assays such as dPCR may be vulnerable to false positive results. To prevent this, we used a stringent and internally validated method for exclusion of false positive results. Equivocal samples were retested with dPCR and in most patients where sufficient DNA material was available the results were duplicated using a separate droplet dPCR assay to ensure the accuracy of our findings.

In our study, a third of the pre-treatment T790M mutations occurred at allele frequencies between 0.01% and 0.1%. This finding is corroborated by other studies, where pre-treatment T790M is generally seen at low frequencies^{14, 16}. This level is below the limit of detection of standard sequencing methods as well as some of the targeted PCR methods routinely used in clinical practice. For example, the limit of detection of T790M mutations utilising ARMS assay is generally >1%¹⁸; however, a wider range of limit of detection, up to 7%, has been reported in some studies²⁹, which may result in an underestimation of the true incidence of pre-treatment T790M. These results support the notion that T790M mutations generally exist at low allele frequencies level in pre-treatment NSCLC samples and therefore sensitive detection methods are required for accurate detection of such small subclones.

Correlation with Clinicopathological Features

In our cohort of patients, the occurrence of pre-treatment T790M mutation did not differ by patient demographic or clinicopathological factors, which is similar to previous studies reporting no significant association between T790M mutations and age, sex or smoking history^{16, 23, 26}. However, one group has observed an association between pre-treatment T790M and advanced stage of disease⁸ and another study has shown a correlation between pre-treatment T790M and larger tumor size¹⁶. We did not specifically examine the relationship between tumor size and the presence of T790M mutation. In addition, our cohort only contained a small number of patients with early stage disease; therefore, the possible association with tumor stage could not be accurately explored.

We did not identify any significant difference in the distribution of pre-treatment T790M mutations among the different EGFR genotypes. Although previous studies have shown an association between pre-treatment T790M status and the common *EGFR* activating mutations¹⁶, especially the L858R missense mutation^{15, 30, 31}, these study cohorts were largely populated by patients of Asian ethnicity or utilized a different molecular method, which possibly contributed to the discrepancy observed.

Clinical significance

In our study, the pre-treatment T790M positive patients had a faster rate of disease progression compared to the T790M negative group, but only in the first five months of treatment. After five months, the rate of disease progression in the T790M positive group slowed down, matching that of the pre-treatment T790M negative group, a novel finding that has not been reported before. The clinical relevance of low-level pre-treatment T790M remains unclear. Most studies, including three previous meta-analyses, have reported an association between pre-treatment T790M mutation and shorter progression free survival in patients with advanced NSCLC receiving a first or second generation EGFR TKI^{9, 10, 14, 15, 22, 25, 32-34}. In one study, detection of low frequency pre-treatment T790M using droplet dPCR was associated with longer progression free survival on first generation TKIs³⁵. All of these studies were retrospective analyses involving small numbers of patients and used a variety of molecular assays. To explain our observation, we speculate that low-level T790M mutations indicate the presence of small subclones of tumor cells with survival advantage in the setting of first-generation EGFR TKI therapy. Such subclones rapidly expand, resulting in faster disease progression early in the course of

treatment. With time, T790M mutations or other resistance mechanisms would develop in the pre-treatment T790M negative group, promoting treatment failure at a rate which eventually reaching the T790M positive group later in the course of disease. Unfortunately, due to retrospective nature of this study and limited serial tumor samples, we were not able to further explore this hypothesis.

Some studies have also demonstrated an association with poorer overall survival in patients with pre-treatment T790M who are treated with first line first generation *EGFR* TKIs ^{25, 34}. Within the entire cohort, we observed a trend towards decreased survival in T790M positive tumour but this did not reach statistical significant. However, when we examined the subset of patients whose best response was disease progression or stable disease (i.e. non-responders), there was a survival advantage favouring the T790M negative group. Besides conferring resistance to *EGFR* TKI therapy, other clinical implications and function of the T790M mutation in NSCLC remain unknown. Our result suggests that *de novo* T790M may be a marker of poor prognosis in non-responders to first generation *EGFR* TKIs.

In our patient cohort, the response rate to first line first generation *EGFR* TKI was 67%, which is comparable to that reported in clinical trials. Notably, this response rate was equally maintained in patients who had low-level pre-treatment T790M mutation. Three other studies using sensitive molecular assays have also reported response rates of 57% to 70% to erlotinib or gefitinib in patients with pre-treatment T790M mutations ^{9, 14, 15}. This contrasts with studies that used standard sequencing techniques which only detected high level pre-treatment T790M mutations in 0% - 2% of *EGFR* mutant NSCLC patients. In these patients treatment with a first generation *EGFR* TKI was ineffective with response rates of only 8% - 14% and the patients had a very short median progression free survival of 2 – 3 months ^{17, 22, 36-40}. In our study, there was only one patient with pre-treatment T790M positive sample detected via Sanger Sequencing, in which dPCR demonstrated a significantly higher allele frequency (28.5%) compared to the rest of the cohort. This patient received first line erlotinib without any response and treatment was ceased after five weeks. The effect of T790M allele frequency on the sensitivity to first generation *EGFR* TKI has been reported in *in vitro* models, where cells that harbored low variant frequency of T790M (1%-10%) displayed similar sensitivity to erlotinib as cells without T790M, and reduced sensitivity to erlotinib was observed when T790M clones made up >25% of the population ⁴¹. Together, such observations suggest that the presence of T790M in a minor clone of tumour cells does not negatively impact the response rate to first generation *EGFR* TKIs and only high T790M frequencies are associated with early treatment resistance.

A small number of patients received osimertinib after disease progression on a first-generation EGFR TKI. Patients with pre-treatment T790M had markedly longer progression free survival compared to those who did not. Due to the small number of cases we were not able to analyse this group in a statistically meaningful fashion. Nevertheless, this finding generates further speculation that there might be a potential difference in the biology and treatment response to osimertinib in tumours with de novo versus acquired T790M mutations that requires further exploration.

This study had several limitations. The retrospective nature of data collection predisposes to selection bias as well as limitations in the measurements of progression free survival and response rate. However, our patient outcomes were consistent with other reports. The retrospective nature of the study also precludes the serial monitoring of T790M status over time and correlation with treatment history. Therefore, additional research in a prospective setting with serial tumor sampling at clinically relevant time points would be of great value in confirming several hypotheses that arose from this study. Our study included only a small number of patients who received osimertinib, which has now become an option for standard first line therapy. As such, the impact of pre-treatment T790M on response to osimertinib would be a relevant topic of research and studies involving a larger number of subjects are required. Lastly, it should be emphasised that highly sensitive assays such as dPCR may be vulnerable to false positive results. To prevent this, all samples were re-tested and the results were duplicated using a separate digital droplet PCR assay to ensure the accuracy of our findings.

Conclusion

This study confirms a relatively high rate of pre-treatment T790M mutation in patients with *EGFR* mutant NSCLC, and supports the notion of clonal expansion following first generation EGFR TKI treatment. In addition, it demonstrates that the majority of pre-treatment T790M mutations occur at very low allele frequencies, often below the limit of detection of conventional DNA sequencing methods; however, it can be reliably detected using highly sensitive assays such as digital PCR. Based on our data, pre-treatment T790M mutation is associated with a significant increased rate of disease progression in the first five months post treatment; although, the overall survival and response to treatment seems to be unaffected. We also observed similar response rates to first line first generation EGFR TKIs in patients irrespective of pre-treatment T790M status, suggesting that tumours with low-level T790M are also

sensitive to these drugs. It would be desirable to establish a clinically meaningful threshold (level of variant frequency) for T790M mutation, that potentially impact prognosis and patient management, via further large-scale prospective studies. The latter would also aid in gaining a deeper understanding of the complexities and clinical relevance of co-existent somatic variants in non-small cell lung carcinoma.

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Figure Legends:

Figure 1. Progression free survival in stage IV patients who underwent first line TKI treatment by A) T790M status and B) T790M allele variant frequency (VF)

Figure 2. Overall survival for stage IV patients treated with first line TKI stratified by pre-treatment T790M status

Figure 1. Progression free survival in stage IV patients who underwent first line TKI treatment by A) T790M status and B) T790M allele variant frequency (VF)

A

B

Figure 2. Overall survival for stage IV patients treated with first line TKI stratified by pre-treatment T790M status

Figure S1. Relative hazard rates of disease progression over time since treatment with first line TKI for stage IV patients with pre-treatment T790M positive tumours (black solid line) relative to patients with pre-treatment T790M negative tumours (grey dashed line at HR=1). Shading represents 95%CI.

Table 1. Allele frequency of pre-treatment T790M in 109 EGFR mutant NSCLC tumour samples.

Allele Frequency	Specimens N (%)
≥10%	1 (0.9)
≥1% - <10%	1 (0.9)
≥0.1 - <1%	27 (24.8)
≥0.01%-<0.1%	15 (13.8)
0%	65 (59.6)

Table 2. Cross tabulation of patient characteristics by pre-treatment T790M status for stage IV patients who underwent first line TKI therapy (n=64)

Characteristics	Patients (n=64)	T790M Neg (n=40, 62.5%)	T790M Pos (n=24, 37.5%)	p-value
Age group (years)				0.621
<60	23 (35.9)	13 (56.5)	10 (43.5)	
60 – 69	24 (37.5)	15 (62.5)	9 (37.5)	
70+	17 (26.6)	12 (70.6)	5 (29.4)	
Mean age (SD)	63 (11.4)	64 (11.1)	62 (12.1)	0.475
Sex				0.308
Male	20 (31.2)	11 (55.0)	9 (45.0)	
Female	44 (68.8)	29 (65.9)	15 (34.1)	
Ethnicity				0.452
Non-Asian	48 (75.0)	32 (66.7)	16 (33.3)	
Asian	16 (25.0)	9 (56.3)	7 (43.8)	
Smoking Status				0.853
Never smoked	33 (51.5)	22 (66.7)	11 (33.3)	

Smoker	29 (45.5)	18 (62.1)	11 (37.9)	
EGFR Genotype				
Exon 19	33 (51.6)	23 (69.7)	10 (30.3)	0.332
Exon 21	24 (37.5)	14 (58.3)	10 (41.7)	0.459
Exon 18	8 (12.5)	5 (62.5)	3 (37.5)	0.922
Exon 20	3 (4.7)	2 (66.7)	1 (33.3)	0.923

SD = standard deviation

Supplementary Data : Establishing the Threshold for True Positive Results

Table S1: Detailed results of dPCR control experiments for EGFR T790M assay by QS3D dPCR
LOB (limit of blank) % VAF (A), FAM copies/μl (B) and FAM events (C)

A

Diagnosis	Sample	DNA input (ng)	Copies/μl VIC	T790M VAF (%)	Mean	Standard Deviation (SD)	LoB VAF (%) [(Mean + (1.645 x SD))]
Normal	Control 1	50	84.2	0	0.02	0.03	0.06
Normal	Control 2	50	166.3	0			
Normal	Control 3	50	226.7	0.04			
Normal	Control 4	50	77.3	0.1			
Normal	Control 5	50	87.4	0			
Normal	Control 6	50	89.2	0.02			
Normal	Control 7	50	64.2	0			
Normal	Control 8	50	165.2	0.02			
Normal	Control 9	50	342.2	0			
Normal	Control 10	50	89.4	0			
Normal	Control 11	50	89.2	0.02			
Normal	Control 12	50	88.7	0			
Normal	Control 13	50	95.6	0.04			
Normal	Control 14	50	84.6	0			
Normal	Control 15	50	83.2	0			

B

Diagnosis	Sample	DNA input (ng)	Copies/μl VIC	Copies/μl FAM	Mean	Standard Deviation (SD)	LoB copies/μl [(Mean + (1.645 x SD))]
Normal	Control 1	50	84.2	0	0.03	0.04	0.09
Normal	Control 2	50	166.3	0			
Normal	Control 3	50	226.7	0.01			
Normal	Control 4	50	77.3	0.08			
Normal	Control 5	50	87.4	0			
Normal	Control 6	50	89.2	0.08			
Normal	Control 7	50	64.2	0			
Normal	Control 8	50	165.2	0.08			

Normal	Control 9	50	342.2	0			
Normal	Control 10	50	89.4	0			
Normal	Control 11	50	89.2	0.08			
Normal	Control 12	50	88.7	0			
Normal	Control 13	50	95.6	0.08			
Normal	Control 14	50	84.6	0			
Normal	Control 15	50	83.2	0			

C

Diagnosis	Sample	DNA input (ng)	Copies/ μ l VIC	FAM Events	Mean	Standard Deviation (SD)	LoB FAM Events [(Mean + (1.645 x SD))]
Normal	Control 1	50	84.2	0	0.4	0.5	1.2
Normal	Control 2	50	166.3	0			
Normal	Control 3	50	226.7	1			
Normal	Control 4	50	77.3	1			
Normal	Control 5	50	87.4	0			
Normal	Control 6	50	89.2	1			
Normal	Control 7	50	64.2	0			
Normal	Control 8	50	165.2	1			
Normal	Control 9	50	342.2	0			
Normal	Control 10	50	89.4	0			
Normal	Control 11	50	89.2	1			
Normal	Control 12	50	88.7	0			
Normal	Control 13	50	95.6	1			
Normal	Control 14	50	84.6	0			
Normal	Control 15	50	83.2	0			

Table S2: Detailed results of dPCR control experiments for EGFR T790M assay by QS3D dPCR
LOD (limit of detection) % VAF (A), FAM copies/μl (B) and FAM events (C).

A

T790M reference DNA expected % mutation	DNA input (ng)	Copies/μl VIC	T790M VAF (%)	Mean	Standard Deviation (SD)	LoD VAF (%) [LoB + (1.645 x SD (low conc. sample))]
0.05%	50	1729.9	0.02	0.02	0.004	0.07
0.05%	50	1714.7	0.01			
0.05%	50	1833.1	0.01			
0.05%	50	1806.4	0.02			
0.05%	50	1786.3	0.02			

B

T790M reference DNA expected % mutation	DNA input (ng)	Copies/μl VIC	Copies/μl FAM	Mean	Standard Deviation (SD)	LoD FAM Copies/μl [LoB + (1.645 x SD (low conc. sample))]
0.05%	50	1729.9	0.304	0.28	0.05	0.18
0.05%	50	1714.7	0.21			
0.05%	50	1833.1	0.241			
0.05%	50	1806.4	0.366			
0.05%	50	1786.3	0.256			

C

T790M reference DNA expected % mutation	DNA input (ng) [Qubit]	Copies/μl VIC	FAM Events	Mean	Standard Deviation (SD)	LoD FAM Events [LoB + (1.645 x SD (low conc. sample))]
0.05%	50	1729.9	4	4	0.0	1.2
0.05%	50	1714.7	4			
0.05%	50	1833.1	4			
0.05%	50	1806.4	4			
0.05%	50	1786.3	4			

Table S3: Detailed results of dPCR control experiments for EGFR T790M assay by Droplet dPCR
LOB (limit of blank) % VAF (A), FAM copies/μl (B) and FAM events (C)

A

Diagnosis	Sample	DNA input (ng)	Copies/□ VIC	T790M VAF (%)	Mean	Standard Deviation (SD)	LoB VAF (%) [(Mean + (1.645 x SD))]
Normal	Control 1	50	1872	0.04	0.01	0.02	0.03
Normal	Control 2	50	1802	0			
Normal	Control 3	50	947	0.08			
Normal	Control 4	50	1572	0			
Normal	Control 5	50	1689	0			
Normal	Control 6	50	1755	0			
Normal	Control 7	50	1872	0			
Normal	Control 8	50	1746	0			
Normal	Control 9	50	2274	0			
Normal	Control 10	50	1727	0			
Normal	Control 11	50	2187	0			
Normal	Control 12	50	1727	0			
Normal	Control 13	50	2187	0			
Normal	Control 14	50	2165	0.03			
Normal	Control 15	50	2355	0			
Normal	Control 16	50	2514	0			
Normal	Control 17	50	1450	0			
Normal	Control 18	50	1437	0			
Normal	Control 19	50	1443	0			
Normal	Control 20	50	1467	0			
Normal	Control 21	50	4159	0			
Normal	Control 22	50	4272	0			
Normal	Control 23	50	4540	0			
Normal	Control 24	50	4491	0.02			
Normal	Control 25	50	4246	0			
Normal	Control 26	50	4945	0.01			
Normal	Control 27	50	3720	0			

Normal	Control 28	50	3989	0			
Normal	Control 29	50	3855	0			
Normal	Control 30	50	3922	0			

B

Diagnosis	Sample	DNA input (ng)	Copies/ μ l VIC	Copies/ μ l FAM	Mean	Standard Deviation (SD)	LoB Copies/ μ l FAM [(Mean + (1.645 x SD))]
Normal	Control 1	50	1872	0.2	0.03	0.09	0.18
Normal	Control 2	50	1802	0			
Normal	Control 3	50	947	0.4			
Normal	Control 4	50	1572	0			
Normal	Control 5	50	1689	0			
Normal	Control 6	50	1755	0			
Normal	Control 7	50	1872	0			
Normal	Control 8	50	1746	0			
Normal	Control 9	50	2274	0			
Normal	Control 10	50	1727	0			
Normal	Control 11	50	2187	0			
Normal	Control 12	50	1727	0			
Normal	Control 13	50	2187	0			
Normal	Control 14	50	2165	0.2			
Normal	Control 15	50	2355	0			
Normal	Control 16	50	2514	0			
Normal	Control 17	50	1450	0			
Normal	Control 18	50	1437	0			
Normal	Control 19	50	1443	0			
Normal	Control 20	50	1467	0			
Normal	Control 21	50	4159	0			
Normal	Control 22	50	4272	0			
Normal	Control 23	50	4540	0			
Normal	Control 24	50	4491	0.1			

Normal	Control 25	50	4246	0			
Normal	Control 26	50	4945	0.1			
Normal	Control 27	50	3720	0			
Normal	Control 28	50	3989	0			
Normal	Control 29	50	3855	0			
Normal	Control 30	50	3922	0			

C

Sample Type	Diagnosis	Sample	DNA input (ng)	Copies/ μ l VIC	T790M FAM Events	Mean	Standard Deviation (SD)	LoB FAM Events [(Mean + (1.645 x SD))]
FFPE	Normal	Normal 7	50	1872	1	0.17	0.4	0.8
FFPE	Normal	Normal 7	50	1802	0			
FFPE	Normal	Normal 7	50	947	1			
FFPE	Normal	Normal 7	50	1572	0			
FFPE	Normal	Normal 7	50	1689	0			
FFPE	Normal	Normal 7	50	1755	0			
FFPE	Normal	Normal 7	50	1872	0			
FFPE	Normal	Normal 7	50	1746	0			
FFPE	Normal	Normal 7	50	2274	0			
FFPE	Normal	Normal 7	50	1727	0			
FFPE	Normal	Normal 9	50	2187	0			
FFPE	Normal	Normal 9	50	1727	0			
FFPE	Normal	Normal 9	50	2187	0			
FFPE	Normal	Normal 9	50	2165	1			
FFPE	Normal	Normal 9	50	2355	0			
FFPE	Normal	Normal 9	50	2514	0			
FFPE	Normal	Normal 9	50	1450	0			
FFPE	Normal	Normal 9	50	1437	0			

FFPE	Normal	Normal 9	50	1443	0			
FFPE	Normal	Normal 9	50	1467	0			
FFPE	Normal	Normal 10	50	4159	0			
FFPE	Normal	Normal 10	50	4272	0			
FFPE	Normal	Normal 10	50	4540	0			
FFPE	Normal	Normal 10	50	4491	1			
FFPE	Normal	Normal 10	50	4246	0			
FFPE	Normal	Normal 10	50	4945	1			
FFPE	Normal	Normal 10	50	3720	0			
FFPE	Normal	Normal 10	50	3989	0			
FFPE	Normal	Normal 10	50	3855	0			
FFPE	Normal	Normal 10	50	3922	0			

Table S4: Detailed results of dPCR control experiments for EGFR T790M assay by Droplet dPCR
LOD (limit of detection) % VAF (A), FAM copies/μl (B) and FAM events (C).

A

T790M reference DNA expected % mutation	DNA input (ng)	Copies/μl VIC	T790M VAF (%)	Mean	Standard Deviation (SD)	LoD VAF (%) [LoB + (1.645 x SD (low conc. sample))]
0.05%	50	912	0.03	0.03	0.005	0.04
0.05%	50	904	0.03			
0.05%	50	924	0.03			
0.05%	50	908	0.02			
0.05%	50	920	0.02			

B

T790M reference DNA expected % mutation	DNA input (ng)	Copies/μl VIC	Copies/μl FAM	Mean	Standard Deviation (SD)	LoD FAM Copies/μl [LoB + (1.645 x SD (low conc. sample))]
0.05%	50	912	0.3	0.24	0.05	0.26
0.05%	50	904	0.3			
0.05%	50	924	0.2			
0.05%	50	908	0.2			
0.05%	50	920	0.2			

C

T790M HD780 reference cfDNA expected % mutation	DNA input (ng) [Qubit]	Copies/μl VIC	FAM Events	Mean	Standard Deviation (SD)	LoD FAM Events [LoB + (1.645 x SD (low conc. sample))]
0.05%	50	912	4	4	0.5	1.6
0.05%	50	904	4			
0.05%	50	924	3			
0.05%	50	908	3			
0.05%	50	920	4			

Table S5. *EGFR* mutation genotypes in pre-treatment NSCLC specimens by Cobas/Sanger sequencing

EGFR Genotype	No. of patients (n=109)
Exon 19 deletion	46
Exon 21 p.Leu858Arg	44
Exon 21 p.Leu861Gln	2
Exon 18 p.Gly719Ala	3
Exon 18 p.Gly719X	1
Exon 18 p.Gly719Ser	1
Exon 20 p.Ser768Ile	1
Exon 20 p.His773dup	1
Exon 20 p.Val769_Asp770insASV	1
Exon 21 p.Leu848Arg plus Exon18 p.Glu709Lys	2
Exon 21 p.Leu858Arg plus Exon 20 p.Thr790Met	1
Exon 21 p.Leu858Arg plus Exon 20 p.Ser768Ile	1
Exon 21 p.Leu858Arg plus Exon 20 p.Arg776Cys	1
Exon 18 p.Gly719Ala plus Exon 18 p.Glu709Ala	1
Exon 18 p.Gly719X plus Exon 20 p.Ser768Ile	1
Exon 18 p.719C plus Exon 20 p.S768Ile	1

Table S6. Hazard ratios for rate of disease progression over time in T790M positive patients compared to T790M negative patients treated with first generation EGFR TKI. Significant difference indicated in bold.

Months	HR	Confidence Interval
1	1.8	0.5 – 6.1
2	2.2	0.9 – 5.0
3	2.2	1.1 – 4.5
4	2.1	1.1 – 4.1
5	2.0	1.1 – 3.8
6	1.8	1.0 – 3.5
7	1.7	0.9 – 2.9
8	1.5	0.8 – 2.9
9	1.4	0.7 – 2.6
10	1.2	0.7 – 2.4
11	1.1	0.6 – 2.2
12	1.0	0.5 – 2.0
13	0.9	0.5 – 1.8
14	0.9	0.5 – 1.7
15	0.8	0.4 – 1.6
16	0.7	0.4 – 1.5
17	0.7	0.3 – 1.4
18	0.6	0.3 – 1.3
19	0.6	0.3 – 1.2
20	0.6	0.3 – 1.2

21	0.5	0.3 – 1.2
22	0.5	0.2 – 1.1
23	0.5	0.2 – 1.0
24	0.4	0.2 – 1.0