Lung Cancer 131 (2019) 86–89

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

The presence and variant allele fraction of *EGFR* mutations in ctDNA and development of resistance

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ARTICLE INFO

Keywords:
EGFR mutation
Non-small cell lung cancer
Resistance
Variant allelic fraction

ABSTRACT

Background: Peripheral blood sampling for detection of EGFR T790M in cell-free circulating tumour (ct) DNA in TKI-resistant *EGFR* mutant (*EGFR*m) lung cancer is now standard. The value of more comprehensive sequencing is unknown.

Methods: Prospective ctDNA analysis in patients with *EGFR*m NSCLC was performed using a next generation sequencing (NGS) panel of regions of 11 genes detecting single nucleotide variants and small insertions/deletions at ≥0.1% variant allele frequency (VAF) was performed. Patients were grouped according to treatment phase, including: (A) pre EGFR-TKI, (B) stable or responding to EGFR-TKI, (C) radiographic progression during EGFR-TKI, and (D) during chemotherapy treatment.

Results: Seventy-two patients with stage IV *EGFR*m NSCLC were enrolled and first blood samples were analysed. Primary sensitizing mutations in del19 or L858R were present in 66 (92%) and uncommon *EGFR*m in 6 (8%). Mutations in ctDNA were found in 53 samples (74%). T790M was detected in 3 of 4 patients with T790M-negative tissue. Other co-occurring *EGFR*m were found in 10 patients (7%) including K745R during first-line osimertinib. TP53 (n = 10), KRAS (n = 1), PI3KCA (n = 1) and ALK (n = 3) gene mutations also were detected. The presence of an *EGFR*m (excluding T790M) was associated with untreated or progressive disease, p = 0.04. In TKI-treated patients without radiologic progression, median progression free survival (PFS) was 10 months versus 2.1 months (HR 2.22, 95% CI: 0.89–5.54, p = 0.08) if an *EGFR*m in ctDNA was detected. If T790M was present in ctDNA, median PFS was 3.0 months versus 9.7 months (HR 4.59, 95% CI: 1.43–14.73, p = 0.005). High % VAF of both *EGFR*m and T790M correlated with inferior PFS (p = 0.01 and p = 0.03 respectively).

Conclusion: In addition to the emergence of resistance mutations, the presence of the primary or co-occurring *EGFR*m in patients receiving EGFR-TKIs may associate with shorter PFS and may be useful in longitudinal analyses of ctDNA to direct therapy.

1. Introduction

An increased understanding of the molecular drivers of oncogenesis and resistance in non-small lung cancer (NSCLC) has led to a rapid expansion in the number of targeted therapies available to patients. Accordingly, molecular diagnostics in NSCLC have rapidly expanded, with guidelines now recommending analysis of multiple biomarkers including *EGFR* and *BRAF* gene mutations and rearrangements in *ALK* and *ROS*.

The most common activating mutations in *EGFR*, del19 and L858R, are predictive of response to tyrosine kinase inhibitors (TKIs) in the advanced setting. In patients receiving first or second generation TKIs, the emergent *EGFR* mutation (*EGFR*m) T790M accounts for 60% of resistant cases. Osimertinib, a third generation TKI selectively inhibits both sensitizing *EGFR*m and *T790M* and has demonstrated improvement in progression free survival (PFS) compared to first generation TKIs in the first-line setting [1,2]. The optimal sequencing of *EGFR*-TKIs remains unclear. Resistance mechanisms to *EGFR*-TKIs are

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https://doi.org/10.1016/j.lungcan.2019.03.019
Received 18 January 2019; Received in revised form 12 March 2019; Accepted 19 March 2019

heterogeneous and multiple aberrations may be present simultaneously [3].

Although tumour tissue genotyping remains the gold standard in detection of driver mutations, testing of circulating cell-free DNA (ctDNA) from liquid biopsies is growing and is particularly useful for patients with insufficient tumour tissue or where there may be substantial risk to the patient from invasive diagnostic procedures. Many next generation sequencing (NGS) panels are available that allow simultaneous detection of multiple genes and mutation types relevant in NSCLC from ctDNA, with one recently approved by the Food and Drug Administration (FDA) as a companion diagnostic in the United States (Oncomine™ Dx Target Test, ThermoFisher, Waltham, MA). While repeat tumour biopsies may help characterise the evolution of resistance in patients receiving targeted therapy, this is challenging in lung cancer patients who often are not well enough to undergo serial biopsies, nor does an isolated biopsy reflect the potential heterogeneity of resistance mechanisms at play. Monitoring levels of cell free circulating tumour (ct) DNA has been shown to correlate with disease response and progression with higher levels of ctDNA emerging at the time of radiographic progression [4,5]. We explored ctDNA levels in patients with stage IV *EGFR*m lung cancer in different phases of therapy, including pre-treatment, during *EGFR*-TKI treatment, upon progression and with chemotherapy.

2. Methods

An ongoing prospective study at the Princess Margaret Cancer Centre is enrolling consenting patients with stage IV *EGFR*m NSCLC for longitudinal blood collection. Patients may enrol at any point in their treatment. In this study, we performed a cross-sectional analysis of 72 patient liquid biopsies using an NGS panel assessing regions of 11 genes (*ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1*, *TP53*) and > 150 hotspots with a limit of detection (LOD) down to 0.1% (Oncomine™ Lung cfDNA Assay; Thermofisher). Conduct of this study was approved by the University Health Network Research Ethics Board.

2.1. Clinical data

Patient and treatment characteristics as well as pathologic information were documented prospectively. Patient outcomes including dates of progression and overall survival (OS) were censored as of January 31st, 2018 for this analysis.

2.2. Blood processing and sequencing

Circulating cell-free DNA (cfDNA) was extracted from the plasma fraction of EDTA blood samples within two hours of collection as recommended [6] (QIAamp Circulating Nucleic Acid Kit; Qiagen, Germantown, MD). 20 ng of cfDNA was tested (Oncomine™ Lung ctDNA Assay; ThermoFisher) with barcoded libraries prepared as per manufacturer's instructions on a liquid handling station (Ion Chef™) and sequenced on the Ion S5 XL System (Ion 530 or 540 Chip; ThermoFisher). Sequencing raw data analysis, alignments and variant calling were processed by Torrent Suite software v5.2 followed by variant filtering and annotation by Ion Reporter software v5.2 (ThermoFisher). Sample batching per NGS run generated a read depth (average 84,000x) appropriate for detection of variants to a lower LOD of 0.1%.

2.3. Statistical analyses

Patients were categorized according to TKI treatment and disease response. Group A (n = 11) included EGFR-TKI naïve patients prior to treatment start. Group B (n = 26) included those receiving EGFR-TKI therapy with stable or responding disease. Group C (n = 27) included those progressing on EGFR-TKI (including third generation agents, n = 5). Group D (n = 8) included those receiving chemotherapy or who were not on active treatment.

Patient demographic, treatment and outcome data were reported using descriptive statistics. The association between the presence of mutations and patient treatment and response status was evaluated using Fischer's exact test. The Cox proportional hazards model was used to evaluate the impact of detected *EGFR* mutations and the percentage allele fraction of *EGFR* mutations on progression free survival (PFS). PFS was calculated from date of blood draw to progression date or last disease assessment if alive, or date of death if died without progression. The presence of additional somatic variants detected using the NGS platform were documented.

3. Results

Over a five-month period (October 2016-February 2017) 72 patients with *EGFR*m NSCLC were enrolled and initial cfDNA samples analysed (n = 72 samples). Most were female (65%) and had common mutations in del19 and L858R (92%). Two patients had exon 18 mutations (G719X), 1 patient a mutation in L861Q and three had exon 20 insertions. Over half of patients were Asian (n = 41, 57%). Most patients (n = 63, 88%) had stage IIIB/IV disease at first diagnosis. The median time since diagnosis of stage III/IV disease was 17.5 months (0.5–76.5 months).

3.1. Patient status on TKI-treatment and the presence of mutations

Mutations in ctDNA were detected in 53/72 patients (74%) (Table 1). This included 9/11 (82%) patients in group A (before starting a TKI), 14/26 (54%) in group B (stable on a TKI, median time on TKI 14.0 months [1.0–49.5]), 24/27 (89%) patients in group C (progressing on a TKI, median time on TKI 16.4 months [3.2–71.0]) and 6/8 (75%) patients in group D (on chemotherapy or surveillance). Variants detected are shown in Table 1. Notably of the 19 patients with no ctDNA detected, 12 (63%) were in group B. The median yield of DNA was 56 ng (range 7.2 ng–2560 ng). Higher DNA yields associated with the presence of ctDNA (p = 0.04).

Of the 27 patients progressing on an EGFR-TKI, 26 had sufficient corresponding tissue for T790M evaluation; the overall concordance between tissue T790M and ctDNA in this cohort was 69% (18/26). The patient without sufficient tissue had T790M detected in blood. Furthermore, T790M was detected in 3 of 4 patients included in group D (on chemotherapy) where prior tissue genotyping had reported the absence of T790M. Other co-occurring *EGFR*m were found in 10 patients (7%) including K745R in a patient receiving first-line osimertinib. *TP53* (n = 10, 14%) and *KRAS* (n = 1, 1%) mutations were also detected. The *KRAS* mutation did not impact response to a first generation TKI in this case (PFS 14 months). A *PI3KCA* (n = 1, 1%) mutation (p.E545 K) was found in one patient on a third generation TKI, in the presence of both the sensitizing *EGFR*m and T790M. In three patients receiving TKIs, one of whom had confirmed progression, ALK (n = 3, 4%) gene mutations were also detected (Table 1).

In patients beginning or receiving an EGFR-TKI (groups A–C, n = 64), the presence of an *EGFR*m (n = 39, 61%) including co-occurring mutations (excluding T790M) strongly associated with group A (8/11, 73%) or group C (20/27, 74%), i.e. patients who were newly diagnosed and TKI naive or those progressing on a TKI vs. those stable on treatment (11/26, 42%), p = 0.04. Of those receiving treatment with a TKI (groups B, C, n = 53), T790M was present in 18 (34%); including 12/27 (44%) patients progressing on a TKI (group C) vs. 6/26 (23%) of those stable on treatment (group B). The difference, however, was not significant (p = 0.15).

3.2. The association between EGFR mutations and PFS

When patients had no evidence of radiologic progression or were

Table 1
ctDNA Results (N = 53) and Variants.

Group Ct DNA detected	Mutation in tissue when ctDNA detected	Primary mutation Detected N, (% of group)	Allele fraction range, %	T790M Detected N, (%)	Allele fraction range, %	Co-occurring EGFR Variants N	Allele fraction range, %	Other variants N	Allele fraction range, %
Group A (9/11) TKI naïve 9/11	L858R (4) Del 19 (5)	8 (73)	0.1-10.6	2 (18)	0.2-1.0	L858R (1)	0.1	KRAS *G12S* (1) TP53 (3)	0.1 0.1-0.6
Group B (14/26) On 1st gen TKI 12/21 On 3rd gen TKI 2/5	Del 19 (6) Del19/S768I(1) L858R (5) L858R/T790M (2)	8 (31)	0.1-9.1	6 (23)	0.1-14.5	L858R (2) E709 K (1) K745R (1)*	0.1-0.2 2.9 0.1	TP53 (3) ALK (2) *Arg1275fs,* *Phe1147s* PIK3CA *E545K* (1)	0.2-7.8 4.6 2.1 0.2
Group C (24/27) Progression on 1st gen TKI 21/22 Progression on 3rd gen TKI 3/5	Del 19/T790M (6) Del 19 (7) L858R/T790M (5) L858R (5) G719S (1)	17(63)	0.6-16.0	12 (44)	0.1-12.5	G719S (1) E709 A (1) L858R (1) Del19 (1)	0.6 2.2 0.1 1.8	TP53 (6) ALK (1) *L1196 M,*	0.2-18.0 0.3
Group D (6/8) T790M-ve on chemo 4/8 Uncommon on chemo 2/8	Del 19 (3) G719X (1) Exon20 in. (2)	4 (44)	0.3-6.8	3 (33)	0.1-2.3	Nil	NA	TP53 (2)	0.6-3.6

* Patient on first line osimertinib.

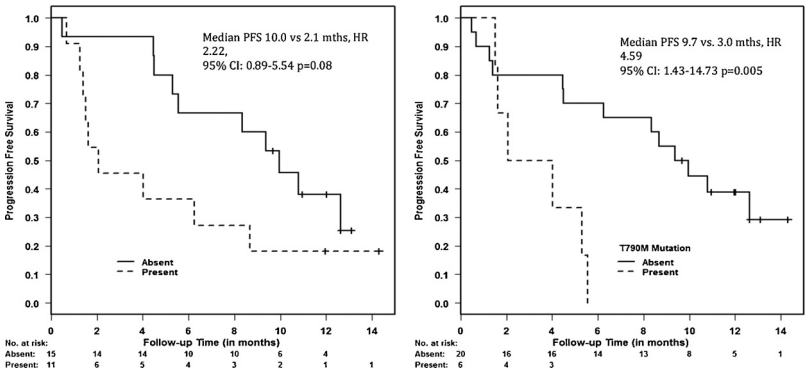

Fig. 1. A/B: PFS associated with (A) presence of primary sensitizing *EGFR* mutation +/- co-occurring EGFRm, and (B) EGFR-T790M resistance mutation in patients on EGFR TKI without radiographic progression (Group B, n = 26).

stable on a TKI (group B) (n = 26), the median progression free survival (PFS), taken from blood draw, was 2.1 months versus 10 months (HR 2.22, 95% CI: 0.89–5.54 p = 0.08) when the primary and/or a co-occurring *EGFR*m was detected in ctDNA, Fig. 1a. One patient in this cohort had an EGFR L858R mutation and an EGFR E709 K mutation detected (% VAF 1.73% and 2.86% respectively). If T790M was present, the median PFS was 3.0 months versus 9.7 months in the absence of a T790M mutation, (HR 4.59, 95% CI: 1.43–14.73 p = 0.005), Fig. 1b.

In univariable regression analyses a high % VAF of the primary/co-occurring *EGFR*m (> median) correlated with inferior PFS compared to a low % VAF or absence of an EGFR mutation (HR = 22.0 in high vs. no mutation, 95% CI: 4.00–120.94, p = 0.004; and HR-0.94 for low vs. no mutation, 95% CI 0.26–3.41, p = 0.92). Similarly a high % VAF of T790M resulted in a shorter PFS compared to the absence of T790M (HR 7.37, 95% CI 1.57–34.53, p = 0.01) Low % VAF also trended toward inferior PFS when compared to the absence of T790M (HR 3.52, 95% CI 0.87–14.20, p = 0.08).

4. Discussion

As the treatment landscape in *EGFR*m NSCLC continues to evolve, resistance mechanisms are becoming better understood. More comprehensive analysis of ctDNA not only overcomes limitations of single site tissue biopsies but may also have an important role in longitudinal analyses, both in monitoring for clearance of primary *EGFR* mutations but also for the emergence of potential subclones and acquired mutations.

While the main focus of research to date has been on the detection of T790M in ctDNA, our analyses show that when stable on treatment with an EGFR-TKI, the presence not only of T790M but other *EGFR* mutations, including co-occurring mutations, predicts a shorter progression free survival. This finding is supported by recent data from AURA3, suggesting that the persistence of an *EGFR*m results in an inferior response to osimertinib [7]. Also, in the small group of patients receiving a TKI in our study the percentage variant allele fraction of the *EGFR*m also predicted time to confirmed progression. Others studies have shown that the % allelic fraction can impact treatment outcomes and survival [8] although this requires validation in well conducted large trials. Furthermore, it remains unknown as to whether the detection of resistance mechanisms prior to confirmatory RECIST progression or the rise in mutation allelic fraction should prompt a change in systemic treatment. The APPLE trial (NCT02856893) will evaluate sequencing of gefitinib and osimertinib and provides an opportunity to assess the impact of switching treatment based on the detection of T790M in ctDNA.

Multi-gene NGS panels (ThermoFisher Oncomine™ Lung ctDNA Research Assay in this study) provide a practical method to characterise resistance mechanisms and to detect variants in multiple genes relevant

in NSCLC including *KRAS, PIK3CA, TP53* and *ALK*. A *PIK3CA* mutation was detected in the presence of T790M in a patient receiving a 3rd generation TKI. Chabon et al using CAPP-seq identified this mutation as a resistance mutation and this has been supported by more recent reports [3,9]. A *de novo KRAS* mutation was discovered in one patient who had a PFS of 14 months on gefitinib. While some studies suggest *EGFR* and *KRAS* mutations are mutually exclusive, others have shown that their co-occurrence does not impact treatment response [10].

The major limitation in this study is the analysis of ctDNA at a single time point in different patients with *EGFR*m NSCLC. However given our findings, we do show the potential utility in ctDNA analysis at any time point in *EGFR*m NSCLC.

5. Conclusion

The presence of any *EGFR* mutation including co-occurring mutations in ctDNA may predict a shorter progression free survival interval. The evaluation of changes in percentage variant allele fraction may be an important biomarker of disease progression and warrants further evaluation.

Disclosures

None relevant.

Conflict of interest

I confirm that the authors listed on this manuscript have no conflicts of interest or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Acknowledgements

This project was supported by the Princess Margaret Cancer Foundation, including through the OSI Pharmaceuticals Foundation Chair (NBL) and the Alan B. Brown Chair (GL).

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