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Identification and monitoring of somatic mutations in circulating cell-free tumor DNA in lung cancer patients

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ABSTRACT

Objectives: Circulating cell-free tumor DNA (ctDNA) isolated from the peripheral blood of non-small-cell lung cancer (NSCLC) patients provides biomarkers for both therapeutic target selection, particularly when direct tumor biopsy is unfeasible, and also for drug resistance monitoring. This study evaluates the reliability and feasibility of ctDNA analysis in an in-house clinical molecular diagnostic workflow.

Materials and methods: Mutation profiling by both standard methods and Next-Generation sequencing (NGS) was carried out and compared on 2 independent lung cancer patient cohorts. Cohort 1 consisted of 50 *EGFR*-mutated NSCLC patients, established on tumour biopsy, for whom ctDNA was collected at disease progression after TKI-inhibitor treatment and could be used to monitor drug resistance. Cohort 2 consisted of 50 newly diagnosed lung cancer patients for whom tumour biopsy was not possible and only ctDNA was available, providing the possibility of biomarker identification.

Results: ctDNA analysis of Cohort 1 verified the persistence of the tumour-detected *EGFR* activating mutation at disease progression by both standard and NGS methods, in 84% and 92% of the cases respectively. The T790M *EGFR* resistance mutation was identified in 71% of the ctDNA *EGFR* mutated samples providing vital information for their disease management. In newly diagnosed Cohort 2 patients, *EGFR* activating mutations were detected in 16% of the patients by both standard and NGS analysis of ctDNA in peripheral blood, providing indication to targeted-therapy otherwise unavailable for this group of patients.

Conclusion: The presented study investigated lung cancer ctDNA analysis, comparing conventional methods versus NGS sequencing, and demonstrated the successful use of plasma ctDNA as a template for targeted NGS tumor gene panel in an in-house routine clinical practice. More importantly, these data underline the advantages of the clinical application of ctDNA NGS analysis for identification of therapeutic targets, real-time monitoring of therapy, and resistance mechanisms in lung cancer patients.

1. Introduction

The identification of actionable alterations has transformed the management of non-small cell lung cancer (NSCLC). Genotype-directed treatment has significantly improved overall survival (OS) in selected patients harboring targetable oncogenic genes [1,2]. Among the most significant findings are the mutations of the *EGFR* gene that identify patients with greater sensitivity to small-molecule tyrosine kinase inhibitors (TKIs).

Significantly, EGFR-TKIs double objective response rate (ORR) and progression free survival (PFS) over cisplatin doublet chemotherapy, and are associated with good tolerability and improved quality of life [3,4]. However, the development of acquired resistance to these agents limits their long-term efficacy. After a median PFS of 10–13 months,

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acquired drug resistance molecular mechanisms emerge, and patients develop tumor recurrence [5]. *EGFR* T790 M somatic mutation is the most frequent alteration detected in approximately half of progressing tumors [6–8].

The third-generation EGFR inhibitor osimertinib has become the standard therapeutic option in *EGFR* T790M-positive patients, progressing on first and second generation EGFR-TKIs [9,10]. Recently, the Phase III FLAURA clinical trial demonstrated that osimertinib significantly prolongs PFS over gefitinib/erlotinib. The presence of baseline *EGFR* T790 M mutation in a subpopulation of patients might represent a biologic interpretation of such results [11], thus opening new questions regarding osimertinib use as up-front therapy in EGFR-mutated NSCLC patients. Moreover, heterogeneous mechanisms of acquired resistance to third generation EGFR-TKIs have been described, including the emergence of *EGFR* C797S mutation [12].

Given the importance of genomic guided treatment at the time of both diagnosis and progression, and the frequent difficulty of obtaining sufficient tissue in patients with advanced stages of NSCLC [4], other sources of tumor DNA than tissues are desperately required for molecular classification, and more importantly, therapy selection.

In such a context, the development of blood-based mutation assays, has shown great potential in providing dynamic and comprehensive genomic profiling of NSCLC in a minimally invasive manner. Also referred to as 'liquid biopsy', these assays analyze circulating cell-free tumor DNA (ctDNA) in the blood of cancer patients [13].

ctDNA is released by passive mechanisms, such as lysis of apoptotic and necrotic cells or digestion of tumor cells by macrophages, and also by active mechanisms. ctDNA shows an enrichment in 150–180 bp fragments, typical of the nucleosomal pattern of DNA fragmentation during apoptosis. Most importantly, ctDNA carries the same somatic alterations as the tumor itself and therefore it can be used to analyze the tumor genotype [14].

Importantly, the results of several studies demonstrated a high concordance rate between mutational *EGFR* profiles in matched tumor and plasma DNA samples, leading both the US Food and Drug Administration and the European Medicines Agency to approve the clinical use of ctDNA for detection of *EGFR* mutations in NSCLC patients if no biopsy material is available [15]. In addition, CAP-IASLC have recently updated their guidelines, recommending the use of ctDNA for advanced NSCLC patients with limited tissue samples [16].

Nevertheless, analysis of ctDNA is challenging due to its high fragmentation and its low fraction of the total circulating cell-free DNA (cfDNA), including that of normal cells [17]. Therefore, standardized methods for ctDNA extraction and analysis are crucial aspects in the setting-up of liquid biopsy molecular diagnostic workflow.

The presented study investigated lung cancer patients ctDNA collection, isolation and analysis, comparing conventional methods (realtime PCR and Digital PCR) versus NGS sequencing, and demonstrated the successful use of plasma ctDNA as template for NGS targeted tumor gene panel analysis in the real world of clinical practice for advanced stage lung carcinoma patients. In order to demonstrate the clinical utility of ctDNA analysis in different management contexts we examined two different cohorts. The first cohort was examined to demonstrate the ability to monitor drug resistance and disease progression after TKI-inhibitor treatment from peripheral blood, whilst in the second cohort, biomarker identification was being performed in treatment naïve patients with new diagnosis for whom histological specimens were unavailable for direct analysis.

2. Materials and methods

2.1. Study cohort

This is a retrospective study, including 100 patients with advanced lung cancer referring to three Institutions: San Raffaele Scientific Institute, Milan (n = 83), Santa Chiara Hospital, Trento (n = 9), University Spedali Civili, Brescia (n = 8).

Patients were divided into two cohorts. Cohort 1: 50 patients with advanced EGFR-mutated NSCLC (Table 1) who had progressed during first- or second-generation EGFR-TKI treatment, according to RECIST criteria (version 1.1), for whom were available both tumor tissue DNA (ttDNA) with a confirmed activating EGFR mutation at diagnosis and cfDNA at disease progression after TKI-inhibitor treatment; Cohort 2:

Table 1
Clinical-pathological characteristics of 50 EGFR-mutated NSCLC patients on progression to EGFR-TKI.

Characteristics		No. of patients n = 50 (100%)
Age		
	Median (range), y	69 (31-89)
Sex		
	Male	21 (42%)
	Female	29 (58%)
Histology		
	Adenocarcinoma	50 (100%)
Stage		
	IIIB	2 (4%)
	IV	48 (96%)
ALK rearrangements		
	Positive	0 (0%)
	Negative	50 (100%)
ROS1 rearrangements		
	Positive	0 (0%)
	Negative	50 (100%)
PD-L1 expression		
	Positive (≥50%)	5 (10%)
	Negative (<50%)	45 (90%)
EGFR activating mutations		
	Exon 19	35 (70%)
	Exon 21	13 (26%)
	Other	2 (4%)
ECOG PS		
	0-1	46 (92%)
	2	4 (8%)
Previous EGFR-TKIs		
	Gefitinib	20 (40%)
	Erlotinib	18 (36%)
	Afatinib	12 (24%)
	Osimertinib (after Gefitinib)	2 (4%)

Abbreviations: ECOG PS; ECOG performance status; EGFR-TKIs EGFR Tyrosine Kinase Iinhibitors.

Table 2
Clinical-Pathological characteristics of advanced lung cancer patients at diagnosis.

Characteristics			No. of patients n = 50 (100%)
Age			
		Median (range), y	81 (42-91)
Sex			
		Male	34 (68%)
		Female	16 (32%)
Diagnosis			
	Histology/citology		25 (50%)
		Adenocarcinoma	21 (84%)
		Other	4 (16%)
	No diagnosis		25 (50%)
Stage			
		IV	42 (84%)
		Other	8 (16%)
Metastases			
		Intrathoracic	25 (50%)
		Extrathoracic	25 (50%)
ECOG PS			
		0-1	11 (22%)
		2	39 (78%)

Abbreviations: ECOG PS, ECOG performance status.

50 lung cancer patients (Table 2) who were either not suitable for tumor biopsy due to co-morbidities, or received a histological/cytological NSCLC diagnosis without an adequate amount of tissue to perform molecular characterization, and for whom cfDNA was available.

A comprehensive written informed consent was signed for the procedures and the diagnostic workup. All the information regarding the human material was managed using anonymous numerical codes. All samples were handled in compliance with the Helsinki declaration. This study was approved by the Institutional Review Board (IRB).

2.2. Blood sample collection

In order to increase the robustness of a ctDNA-based biomarker test design, it is of key importance to both minimize ctDNA degradation in the blood collection tube and prevent the wild-type genomic DNA release from cell apoptosis and/or lysis of nucleated blood cells. To this end Streck Cell-Free DNA BCT-tubes (Streck, Omaha, NE), which contain cell-stabilizing agents that prevent cell lysis, were used to collect venous blood from all the 100 patients.

Plasma was separated from the cellular fraction by centrifugation at 1600 (± 150) g for 10 min; plasma supernatant was then centrifuged at 3000(± 150) g for 10 min and stored at -80 C until DNA extraction.

2.3. DNA extraction

Circulating Free DNA was extracted from 4 ml of plasma by using QiAamp MinElute ccfDNA Kits (Qiagen, Hilden Germany) according to the supplier's instructions. Elution was performed in 60 µl and isolated cfDNA was kept at −20 °C. cfDNA was quantified by using the High Sensitivity DNA Assay on the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA). Overall, the median cfDNA concentration in plasma samples was 600 pg/ul.

The genomic DNA from NSCLC tissue samples (ttDNA) was isolated by automated extraction using the Magcore Nucleic Acid Extractor (RBC Bioscience, by Diatech Pharmacogenetics, Jesi Italy) following the manufacturer's protocols. Quantity of isolated DNA was assessed by Qubit 3.0 Fluorometer (Thermo Fisher Scientific).

2.4. EGFR mutational status by standard methods

EGFR status was analyzed using cfDNA for all the 100 patients of the study by standard methods: real-time PCR was performed by using both Easy EGFR Kit CE IVD (Diatech Pharmacogenetics) and AmoyDx - EGFR 29 Mutations Detection Kit (Amoy Diagnostics, by LCM-Genect, Milan, Italy), following manufacturer's instructions on QuantStudio5 instrument (Thermo Fisher Scientific). For *EGFR* T790 M detection digital droplet PCR (RainDance Technologies, by Diatech Pharmacogenetics) was also performed: PCR primers, hydrolysis probes and amplification conditions were implemented as previously described [18]. Droplet counts were determined using the RainDrop Analysis software.

EGFR status on ttDNA was analyzed as previously described [19].

2.5. Mutation profiling by Next-generation sequencing (NGS) of ctDNA

Somatic alterations that involve driver oncogenes in lung carcinoma, were examined by Oncomine™ Lung cfDNA assay (Thermo Fisher Scientific) on IonTorrent S5 (Thermo Fisher Scientific). The assay analyses 160 mutations in 11 genes implicated in lung cancer (*ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1*, and *TP53*) and detects somatic variants at low frequency (MAF 0.1% with 20 ng cfDNA input). Targeted libraries were prepared following the user guide instructions and were sequenced on the 520™ Chip. Raw data analyses, base calling and alignment were performed using Torrent Suite v5.4. Oncomine Liquid Biopsy DNA filtering was applied in Variant Caller v.5.4 (VC) to detect DNA changes when compared to reference genome hg19 (build 37). Identified mutations were further checked by manual inspection of BAM files using the Integrative Genomics Viewer (IGV) 2.3 (Broad Institute, Cambridge, MA).

2.6. FISH analysis

MET gene copy-number (CN) status was assessed by Fluorescence In Situ Hybridization (FISH) on formalin-fixed, paraffin-embedded tissue 4µm tumor tissue sections using a dual-color FISH probe set (ZytoLight ® SPEC MET/CEN 7 Dual Color Probe, Zytovision, Germany), following manufacturer's protocols. Slides were analyzed using Nikon 90i fluorescence microscope (Nikon Instruments SpA, Italy) with both a single-pass (green and orange) and a triple-pass filter band [4′,6-diamidino-2-phenylindole (DAPI)/green/orange]; images were captured by Genikon software (Nikon). MET CN and the number of CEN7 copies per nucleus were counted in at least 60 tumor cells and the mean MET/CEN7 ratio was calculated for each specimen. A sample was considered to have *MET* amplified if the mean MET/CEN7 ratio was ≥ 2.0 or, if the MET/CEN7 ratio was < 2.0, the *MET* CN was ≥ 6 copies/cell or *MET* signal clusters were seen in more than 10% of tumor cells [20].

2.7. Statistical analysis

Medical records were reviewed and data extracted on both clinical/pathologic features and treatment histories. Clinical data were available for patients referring to San Raffaele Scientific Institute, only. PFS was measured from the time of treatment initiation to clinical/radiographic progression. The Kaplan-Meier method was used to estimate all PFS endpoints. Patients and tumor characteristics were summarized using descriptive statistics. Statistical analysis was performed using GraphPad PRISM version 5.04 for windows (Graph Pad Software, San Diego, CA).

3. Results

3.1. Mutational status of NSCLC patients at disease progression after EGFR –TKI treatment and clinical implication

We identified 50 *EGFR*-mutated NSCLC patients who had progressed during *EGFR*-TKI treatment. Baseline clinical and pathological characteristics of these patients are summarized in Table 1.

At the time of NSCLC diagnosis, ttDNA EGFR analysis identified 35 (70%) mutations in exon 19, 13 (26%) in exon 21, 1 (2%) in exon 18, and 1 (2%) in exon 20. Twenty patients received gefitinib, 18 erlotinib, and 12 afatinib as first line treatment for metastatic disease. Among the 33 patients with available medical history, 28 (85%) had a partial response, 1 (3%) a complete response, 1 (3%) a stable disease, and only 3 (9%) developed disease progression at the first tumor assessment. Median PFS was 12.5 months.

At progression, both the persistence of initial TKI-activating EGFR mutations and the onset of T790 M drug resistance mutation were investigated in ctDNA by both standard methods and NGS approach.

ctDNA analysis at disease progression by standard methods showed the persistence of the initial *EGFR* activating mutation in 42 out of 50 cases. The concordance rate between ttDNA *EGFR* status at diagnosis and ctDNA at progression was 84%, with a specificity of 100% (Table 3). In 30 (71%) out of 42 ctDNA mutated samples, the *EGFR* T790 M resistance mutation was identified; the 8 samples with no *EGFR* activating mutations on ctDNA analysis were also negative for the *EGFR* resistance mutation (Table 3).

NGS ctDNA analysis at disease progression by the Oncomine™ Lung cfDNA assay was possible in 49 out of 50 cases, demonstrating the high feasibility of such approach (98% technical success rate), with a practical turnaround time of 4 working days.

Forty-five *EGFR* mutated ctDNAs out of the 49 analyzed samples were observed (Table 3), with a concordance rate of *EGFR* activating status between ttDNA at diagnosis and ctDNA at progression of 92%, and a specificity of 100%.

Table 3
EGFR activating and resistance mutations in 50 NSCLC patients on progression to EGFR-TKI before treatment (ttDNA) and at progression (ctDNA).

Sample	Activating mutations			Resistance mutations	
	ttDNA by standard methods	ctDNA by standard methods	ctDNA by NGS	ctDNA by standard methods	ctDNA by NGS
1	*E709K+G719A*	+	+	+	+
2	*delE746-A750*	+	+	+	+
3	*delE746-A750*	+	+	+	+
4	*delE746-A750*	+	+	+	+
5	*delE746-A750*	+	+	+	+
6	*delE746-A750*	+	+	+	+
7	*delE746-A750*	+	+	+	+
8	*delE746-A750*	+	+	+	+
9	*delE746-A750*	+	+	+	+
10	*delE746-A750*	+	+	+	+
11	*delE746-A750*	+	+	+	+
12	*delE746-A750*	+	+	+	+
13	*delE746-A750*	+	+	+	+
14	*delE746-A750*	+	+	+	+
15	*delE746-A750*	+	+	+	+
16	*delE746-A750*	+	+	+	+
17	*delE746-A750*	+	+	+	+
18	*delE746-A750*	+	+	+	+
19	*delE746-A750*	+	+	+	+
20	*delL747-T751*	+	+	+	+
21	*delL747-T751>P*	+	+	+	+
22	*delL747-T751>P*	+	+	+	+
23	*delE746-T751>VA*	+	+	+	+
24	*delT751-I759>N*	+	+	+	+
25	*L858R*	+	+	+	+
26	*L858R*	+	+	+	+
27	*L858R*	+	+	+	+
28	*L858R*	+	+	+	+
29	*L858R*	+	+	+	+
30	*delE746-A750*	+	+	-	+
31	*L858R*	+	+	-	+
32	*D770-N771>QRG*	+	+	-	-
33	*L858R*	+	+	-	-
34	*L858R*	+	+	-	-
35	*L858R*	+	+	-	-
36	*K745-E746>IPVAIK*	+	+	-	-
37	*L858R*	+	+	-	-
38	*L858R*	+	+	-	-
39	*delE746-A750*	+	+	-	-
40	*delE746-A750*	+	+	-	-
41	*delL747-T751>P*	-	+	-	+
42	*L858R*	-	+	-	-
43	*delE746-A750*	-	+	-	-
44	*L858R*	-	+	-	-
45	*delE746-A750*	-	+	-	-
46	*delE746-A750*	-	-	-	-
47	*delE746-T751>I*	-	-	-	-
48	*delI747-A750>P*	-	-	-	-
49	*delE746-A750*	+	-	-	-
50	*L858R*	+	/	+	/

Abbreviation: ttDNA, tumor tissue DNA; ctDNA, circulating tumor DNA; +, detected mutation; -, not detected mutation; /, not available.

On ctDNA samples, concordance rate between standard and NGS analysis was 88% (43 samples out of 49): 5 of the 6 discordant cases were *EGFR* mutated by NGS (same *EGFR* mutation of ttDNA); 1 case was *EGFR* wild type by NGS (Table 3).

EGFR T790 M resistance mutation was identified by NGS in 32 (71%) out of 45 mutated ctDNA samples. *EGFR* T790 M mutation concordance rate between standard methods and NGS was 94% (46 out 49). Three cases were discordant: all of them mutated by NGS and wild-type by standard methods (Table 3).

Liquid biopsy was performed at the time of progression on osimertinib in 2 patients and in both of them an acquired C797S mutation was found in *cis* with the T790 M mutation by NGS.

Somatic mutations other than *EGFR* mutations were detected in 27 patients out of 49. In particular, mutations in *TP53* (22%), *PIK3CA* (16%), *KRAS* (8%), *ALK* (8%), *ROS1* (8%), *BRAF* (6%), *NRAS* (4%), *ERBB2* (2%), and *MP2K1* (2%) were found (Fig. 1A).

In order to better understand resistance mechanisms at time of progression, 8 patients who were ctDNA *EGFR*-T790 M negative by both standard and NGS analysis, underwent tumor tissue re-biopsy. *EGFR* T790 M status and *MET* gene amplification analysis were performed. Only one sample was T790 M mutated (concordance rate between ctDNA and re-biopsy tDNA 87.5%). *MET* gene amplification was found in 4 patients, a transformation to small cell lung cancer was observed in 1 case, and no additional molecular alterations were found in 2 cases.

Osimertinib was administered to the 30 patients who were *EGFR* T790 M positive by standard methods. At the time of manuscript preparation, complete clinical data were available for 14 out of these 30 patients. Whilst treatment is ongoing we have already observed 1 patient with complete response, 5 patients with partial response, 4 patients with stable disease, and 4 patients with progressive disease.

Interestingly, in two patients at progression a dissociated metabolic response was observed. One patient developed partial remission in 4 out of 5 liver lesions, and metabolic progression of one lesion. We performed a liver biopsy of the progressive lesion that resulted *EGFR* T790 M negative and *MET* gene amplification positive. A similar situation was found for the other patient: complete response to brain lesions and progressive disease on mediastinal lymph nodes; again, the tumor biopsy of progressive lymph nodes showed the presence of *MET* gene amplification and no *EGFR* T790 M mutation (Fig. 2).

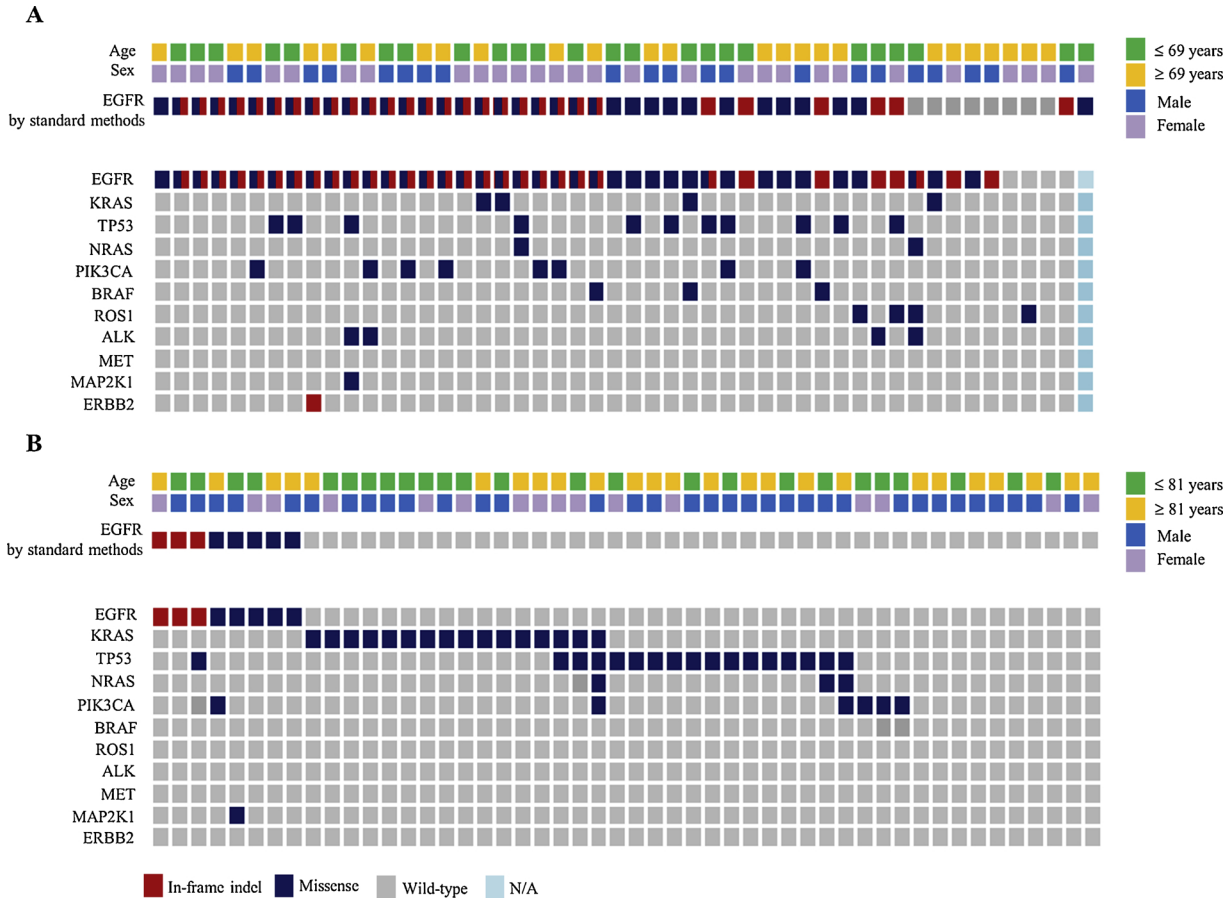

Fig. 1. Heat map of somatic mutations identified in the 11 genes sequenced in ctDNA from plasma of the two courts of patients: (**A**) NSCLC patients on progression on EGFR-TKI; (**B**) advanced lung cancer patients at diagnosis.
The colors indicate the different types of mutations found (see color key).

3.2. Mutational status of advanced lung cancer patients at diagnosis and clinical implication

We identified 50 advanced lung cancer patients, whom were either unsuitable for tumor biopsy, due to co-morbidities, or received a histological or cytological diagnosis of NSCLC without adequate amount of tissue to perform molecular characterization. ctDNA was collected for all patients. The baseline clinical and pathologic characteristics of these patients are summarized in Table 2. Half of the patients had a cytological/histological diagnosis of NSCLC of which there were 21 adenocarcinomas, 2 squamous carcinomas, 1 NSCLC, 1 large cell neuroendocrine tumor. Median age was higher in this group of advanced lung cancer patients at diagnosis compared to the previously described group of NSCLC patients at progression ($p < 0{,}0001$).

The analysis of ctDNA by standard methods showed the presence of *EGFR* activating mutations in 8 (16%) out of 50 patients: 3 deletions in exon 19, 4 point mutations in exon 21, and 1 point mutation in exon 18.

NGS ctDNA analysis was possible in all 50 cases (100% technical success rate). The same *EGFR* mutations were found in the 8 patients who were *EGFR* positive by standard methods (concordance rate between standard and NGS analysis was 100%). Somatic mutations other than *EGFR* mutations were detected in 35 patients out of 50. In particular mutations in *TP53* (34%), *KRAS* (32%), *PIK3CA* (12%), *NRAS* (6%), and *MP2K1* (2%) were found (Fig. 1B).

Based on these findings, 7 out of 8 patients received treatment with gefitinib: partial responses were observed in all the 7 cases; one patient was not able to continue treatment due to worsening of clinical conditions. Median PFS was 8 months.

4. Discussion

In this study we report the advantages of the clinical application of ctDNA molecular characterization in NSCLC as a pivotal tool for real-time monitoring of therapy, risk of relapse, resistance mechanisms and identification of therapeutic targets. Although not a replacement for a diagnostic tissue biopsy, the minimal-invasive liquid biopsy is becoming more attractive to classify patients according to their mutational profile [21] and the use of ctDNA extracted from peripheral blood plasma is currently the main source for this application [22].

In particular, we investigated the collection, isolation and analysis of plasma ctDNA either in patients at disease progression after TKI-inhibitor treatment to monitor drug resistance, or in treatment naïve patients with new diagnosis for whom histological specimens were unavailable to identify therapeutic targets. For this latter group of patients, ctDNA analysis showed the presence of *EGFR* activating mutations in 16% of patients who could then be referred to TKI therapies, allowing them to receive a customized and effective treatment, with a good tolerability profile. Of note, the median age of patients who underwent liquid biopsy at the time of diagnosis was 78 years compared to 66 years in patients whose ctDNA analysis was performed at progressive disease during TKIs. This suggests that liquid biopsy represents a valid method to detect druggable molecular alterations in frail patients who otherwise could not receive treatment because of the lack of ttDNA.

We demonstrated the successful use of plasma ctDNA for mutation detection by both standard technologies and NGS, proving the NGS-based liquid biopsy diagnostic role in a clinical setting, following a

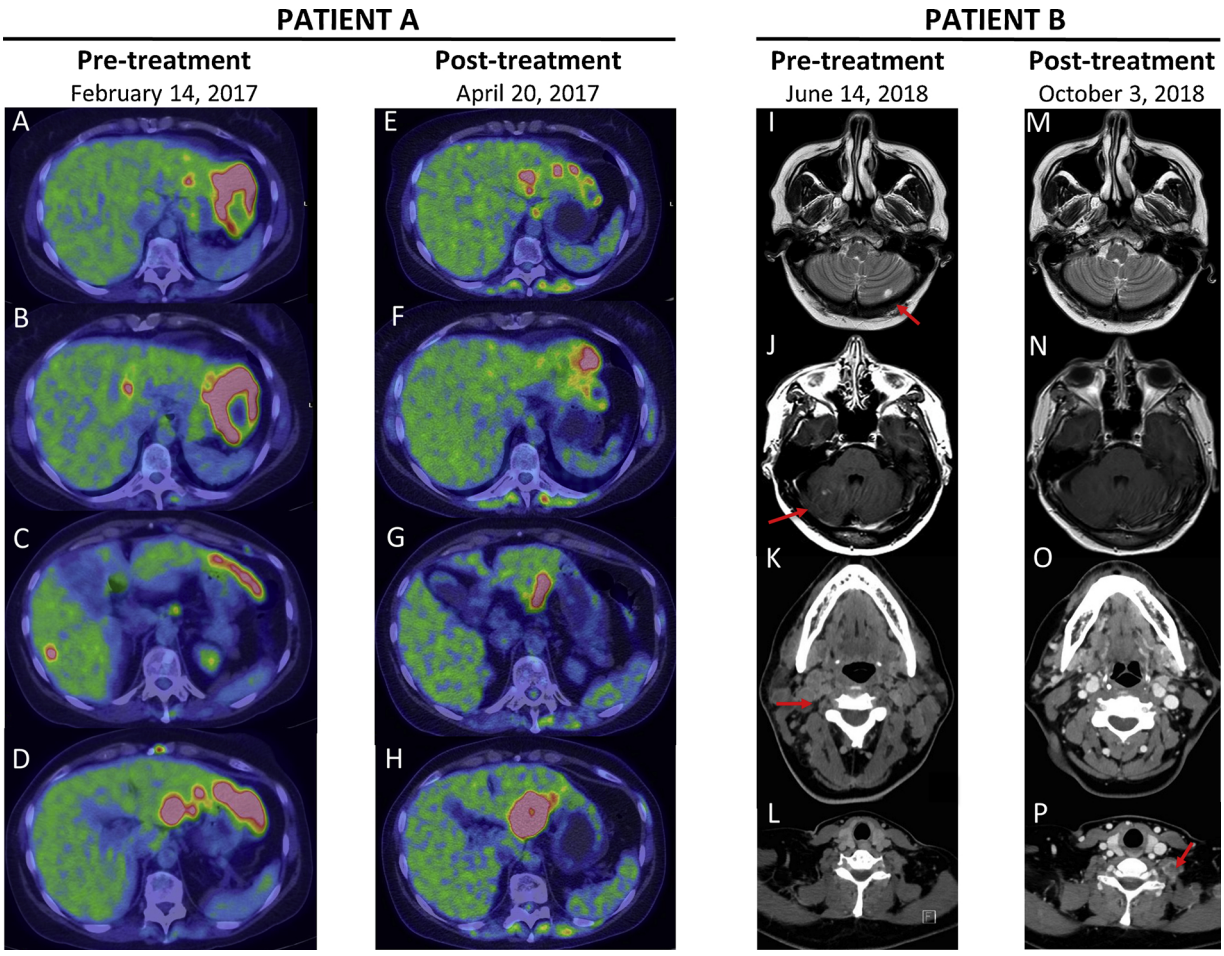

Fig. 2. Representative PET-CT scan images of 2 NSCLC patients with dissociated metabolic response after osimertinib treatment. **Patient A**: Liver lesions with partial metabolic response in **E**, **F**, **G**, in comparison to **A**, **B**, **C**. The lesion with metabolic progression in **H** in comparison to **D** underwent liver biopsy. The biopsy of the progressive lesion was *EGFR* T790 M negative and *MET* gene amplification positive. **Patient B**: Radiologic response on brain lesions in **M**, **N** in comparison to **I**, **J**. The patient had a complete radiologic response at brain. She suffered from multiple brain lesions, not treated with brain radiotherapy. Partial response at lymph nodes site shown in **O**, **P** compared with **K**, **L**. Progression on paraspinal lesion in **P**, that underwent biopsy. The biopsy of the progressive lesion was *EGFR* T790 M negative and *MET* gene amplification positive.

completely in-house workflow. Several examples of NGS based lung ctDNA genotyping has already been reported [23–25] but routine clinical practice reports using in-house workflows are still limited, and more data are needed in order to expand the use of ctDNA NGS analysis in oncology molecular diagnostics. One of the goals of our study was to generate a robust, reproducible and cost-efficient approach of ctDNA genotyping for our clinical setting, thus providing an alternative choice to outsourcing ctDNA analysis that generally requires long turnaround times. Indeed, the total process time for our NGS assay, from blood sample receipt to result reporting, was as short as 4 days. Furthermore the bioinformatic tools, the standardized protocols, and analysis workflows provided by the Oncomine™ Lung cfDNA assay make data evaluation and interpretation feasible for a diagnostics molecular laboratory to implement in routine clinical practice.

Our NGS approach on ctDNA was very promising, showing a high technical success rate (98%), a strong concordance between *EGFR* sensitizing mutations in tissue samples at diagnosis and in plasma at progression (92%), and a high detection rate of plasma T790 M at progression (71%) with a concordance rate between standard methods and NGS of 94%. These results are in line with previously reported data, giving further credibility to the application of in-house non-invasive molecular profiling of lung cancer patients [26,27].

Among the *EGFR* T790 M positive patients who received osimertinib, four showed progressive disease. In two of them a dissociated radiological response was observed. The tissue biopsies of the progressive lesions were *EGFR* T790 M negative and *MET* gene amplification positive, providing additional evidence of NSCLC tumor heterogeneity as already documented in both autopsy and ctDNA-based studies [28].

Our data also support the use of a targeted multigene panel NGS assay for a more informative characterization of the most common mechanisms, other than T790 M mutation, involved in the resistance to third-generation EGFR-TKI [29–31], such as C797S *EGFR* mutation allelic conformation. The exon 20 *EGFR* C797S represents an acquired resistance mechanism to third-generation TKIs as it prevents their binding to the EGFR active site. Such occurrences can be found either in the same (in *cis*) T790M-mutated allele or in the other allele (in *trans*) and, both in vitro and in vivo studies reported that, when C797S emerged in *trans* of the T790 M allele, tumors remain sensitive to first- and third generation EGFR-TKI combinations, whereas tumors remain broadly resistant if C797S emerged in the *cis* position of T790 M allele [32,33].

NGS genotyping of ctDNA at progression identified also additional tumor associated alterations in genes such as *TP53*, *PIK3CA*, *KRAS*, *ROS1*, *BRAF*, *ALK*, *NRAS*, *ERBB2*, and *MP2K1*. These data deserve further investigation in larger data sets, as they can contribute to better define the co-occurring genetic alteration role in affecting the response to TKI EGFR inhibitors. Indeed, heterogeneous resistance mechanisms

of T790M-mutant subclones coexisting with subclones harboring different acquired alterations, including *KRAS* mutations have been described [28,34,35]. Further, distinct combinations of *TP53* mutations with other gene alterations were proved to be major determinants of the tumor immune profile and of the expression of PD-L1 by malignant cells in lung cancer [36]. In particular mutations of genes such as *TP53* may alter the immune landscape via the generation of neoantigens which have been proposed as biomarkers to predict therapeutic effects of immune checkpoint blockade therapy and as potential targets for cancer immunotherapy [37].

Among the various mechanisms that lead to acquired resistance to TKIs therapies, the histologic transformation from adenocarcinoma to SCLC has been observed in 3–20% of patients. This resistance mechanism can be formally proved only by recurrence biopsy. Nevertheless, *TP53* and *RB1* alterations can be used as an indication of this resistance mechanism. In fact, though the molecular basis of this histology transformation are not fully understood, *TP53* and *RB1* gene inactivation has been described in SCLC and therefore associated to histology transformation from adenocarcinoma to SCLC [38,39].

Though economic issues may hinder a NGS approach for ctDNA analysis, it must take into account that NGS molecular profiling allows detection of a wider range of mutations that can be translated into clinically actionable results. Indeed, a multigene NGS analysis can be nowadays affordable and less expensive than multiple single-gene investigations by standard methods.

Overall, a non-invasive diagnostic approach that identifies multiple concomitant resistance mechanisms can have important implications for selection of correct combination therapies targeting the activated signaling pathways. Although the amount of ctDNA recovered from bloodstream cfDNA can be very low, the correct use of this sensitive and targeted NGS technology allows the full exploitation of ctDNA potentiality.

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Declaration of conflict of interest

None declared for all the authors.

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