Original Article

Prospective analysis of liquid biopsies of advanced non-small cell lung cancer patients after progression to targeted therapies using GeneReader NGS platform

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Background: In a significant percentage of advanced non-small cell lung cancer (NSCLC) patients, tumor tissue is unavailable or insufficient for genetic analyses at time to progression. We prospectively analyzed the appearance of genetic alterations associated with resistance in liquid biopsies of advanced NSCLC patients progressing to targeted therapies using the NGS platform.

Methods: A total of 24 NSCLC patients were included in the study, 22 progressing to tyrosine kinase inhibitors and two to other treatments. Liquid biopsies samples were obtained and analyzed using the GeneRead™ QIAact Lung DNA UMI Panel, designed to enrich specific target regions and containing 550 variant positions in 19 selected genes frequently altered in lung cancer tumors. Previously, a retrospective validation of the panel was performed in clinical samples.

Results: Of the 21 patients progressing to tyrosine kinase inhibitors with valid results in liquid biopsy, NGS analysis identified a potential mechanism of resistance in 12 (57%). The most common were acquired mutations in *ALK* and *EGFR*, which appeared in 8/21 patients (38%), followed by amplifications in 5/21 patients (24%), and *KRAS* mutations in one patient (5%). Loss of the p.T790M was also identified in two patients progressing to osimertinib. Three of the 21 (14%) patients presented two or more concomitant alterations associated with resistance. Finally, an *EGFR* amplification was found in the only patient progressing to immunotherapy included in the study.

Conclusions: NGS analysis in liquid biopsies of patients progressing to targeted therapies using the GeneReader platform is feasible and can help the oncologist to make treatment decisions.

Keywords: Non-small cell lung cancer (NSCLC); progression; liquid biopsies; GeneReader

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Introduction

Acquired resistance is an unavoidable process during treatment of cancer patients with targeted therapies. In the case of first and second generation *EGFR*-TKIs, *EGFR* mutant (*EGFR*-mut) non-small cell lung cancer (NSCLC) patients usually progress after a median period of 10–12 months (1). The molecular mechanisms responsible for this process have been extensively investigated and the T790M secondary mutation has emerged as the most frequent resistance-associated molecular alteration in *EGFR*-mut patients, with prevalence around 50–60% (1-3), followed by amplification of membrane receptors, such as *MET* proto-oncogene (MET), erb-b2 receptor tyrosine kinase 2 (*ERBB2*) or fibroblast growth factor receptor 1 (*FGFR1*) (4-7). Other resistance mechanisms include epithelial to mesenchymal transformation (EMT) or histological transformation to small cell lung cancer (SCLC) (4). Third generation EGFR-TKIs are active against tumors with the T790M secondary mutation and one of them, osimertinib, has been approved in this setting (4). However, similarly to first and second line TKIs, patients ultimately progress. Mechanisms of resistance to osimertinib include loss of the T790M (5), emergence of a "tertiary" mutation in exon 20 of *EGFR* (C797S) (8-11), *MET* and *ERBB2* amplifications and *de novo* mutations in *KRAS* (6,12).

In the case of *EML4-ALK* positive patients, disease progression occurs after a median of 9–18 months of treatment with *ALK*-TKIs (13-17). Different mechanisms of acquired resistance have been identified including emergence of *ALK* secondary mutations, which can be detected in 30% of patients, activation of the *EGFR* signaling pathway, *KRAS* mutations and others (18,19). Finally, EMT and SCLC transformation have also been described in this setting (20,21). In contrast with *EGFR*, the spectrum of *ALK* mutations associated with resistance to *ALK*-TKIs is very heterogeneous, being the most common L1196M, G1269A, F1174LC/L, C1156Y and G1202R (22). Importantly, second and third generation ALK-TKIs show different efficacies depending on the type of secondary mutation (23).

Genetic analysis of somatic alterations is mandatory in advanced NSCLC at presentation and progression to targeted agents, in order to select the optimal treatment strategy. However, around 5–20% of patients cannot be biopsied baseline or the tumor tissue in biopsies or cytological samples is insufficient for successful genetic analysis (24,25). This percentage is significantly higher in patients progressing to targeted therapies, where the availability of rebiopsies is limited. In consequence, liquid biopsy samples are of particular relevance in this setting as a surrogate of surgical biopsies for genetic testing (26).

Here, we present the results of the NGS analysis in liquid biopsy samples of NSCLC patients obtained at progression to different therapies using the GeneRead^{TM} QIAact Lung DNA UMI Panel. Our results demonstrate the usefulness of NGS for the detection of genetic alterations associated with resistance in liquid biopsies and, consequently, for the selection of subsequent lines of therapy.

Methods

Sample selection and processing

For the validation of the panel, a total of 45 samples were retrospectively analyzed. They included 20 FFPE samples, 10 paired FFPE/blood samples and 5 cell lines included in paraffin blocks. Patient samples comprised a majority of NSCLC (n=27/30; 90%), but also colorectal (n=1), melanoma (n=1) and ovary (n=1) specimens. All blood and tissue samples had been previously genotyped by non-NGS methodologies, namely PNA-Q-PCR (27), Sanger sequencing or FISH, and were selected to represent a variety of clinically relevant mutations.

For prospective analysis, liquid biopsies of 24 NSCLC patients were obtained at progression to different therapies. They included peripheral blood (n=18, two patients with paired blood-other fluids), pleural fluid (PF, n=3), cerebrospinal fluid (CSF, n=4) and ascites (AF, n=1). Peripheral blood (10 mL) was collected in Vacutainer tubes (BD, Plymouth, UK) and centrifuged at 2,300 rpm for 10 min. The supernatant was then transferred into a new tube and submitted to a second centrifugation immediately followed by cfDNA purification or storage at –20 ℃.

Other fluids (1–10 mL) were processed following the same protocol, cytological extensions of the first and second pellets (sometimes not visible) were performed to evaluate positivity for malignant cells and absence of remaining cells in the final preparation, respectively.

Studies were conducted in accordance with the Declaration of Helsinki under an approved protocol of the institutional review board of the Quirón Hospitals, and de-identified for patient confidentiality. Informed written consent was obtained from all subjects.

cfDNA purification

Purification of cfDNA was performed from 4 mL of fluids using a custom protocol with the QIAsymphony® DSP Virus/Pathogen Midi Kit using a QIAsymphony robot (QIAGEN, Hilden, Germany) and following the manufacturer's instructions. The final elution volume was 50 µL per sample. For liquid biopsies with less than 4 mL, an alternative custom protocol using 1.2 mL and a final elution volume was 30 µL was used. For DNA purification from FFPE samples, the GeneRead DNA FFPE Kit (QIAGEN, Hilden, Germany) was employed, following the manufacturer's instructions. DNA concentration was measured by Qubit®. Samples with ≥2.5 ng DNA/mL were diluted to achieve this concentration.

NGS sample preparation, sequencing run and data processing

NGS was performed with the GeneReader Platform® (QIAGEN, Hilden, Germany). Purified DNA (16.75 µL) was used as a template to generate libraries for sequencing using the GeneRead™ QIAact Lung DNA UMI Panel, according to manufacturer's instructions. The panel is designed to enrich specific target regions containing 550 variant positions in 19 selected genes frequently altered in lung cancer tumors (*AKT1*, *ALK*, *BRAF*, *DDR2*, *EGFR*, *ERBB2/HER2*, *ESR1*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *NTRK1*, *PDGFRA*, *PIK3CA*, *PTEN*, *ROS1*, *FGFR1* and *RICTOR*), including *MET* exon 14 skipping mutations. The panel can also detect copy number variations (CNV) in five genes (*EGFR*, *FGFR1*, *ERBB2/HER2*, *MET*, *RICTOR*).

Libraries were quantified using a QIAxcel® Advanced System, diluted to 100 pg/µL and pooled in batches of 6 (liquid biopsies) or 12 (tissues). Clonal amplification was performed on 625 pg of pooled libraries by the GeneRead Clonal Amp Q Kit using the GeneRead QIAcube and an automated protocol. Following bead enrichment, pooled libraries were sequenced using the GeneRead UMI Advanced Sequencing Q kit in a GeneReader instrument.

QIAGEN Clinical Insight Analyze (QCI-A) software was used to performed the secondary analysis of FASTQ reads, align the read data to the hg19 reference genome sequence, call sequence variants and generate a report for visualization of the sequencing results. Variants were imported into the QIAGEN Clinical Insight Interpret (QCI-I) web interface for data interpretation and generation of final custom report.

Results

Validation of the panel

The NGS workflow using GeneReader requires approximately 5 days from DNA extraction to a final clinical report and allows processing of up to 36 samples per run, with relatively low hands-on time (*Figure 1*). In order to implement the GeneReader Platform together with the QIAact Lung DNA Panel in routine clinical practice, a retrospective validation study was performed. A total of 45 clinical samples, previously genotyped by other methodologies, were selected for the validation cohort, including FFPE tumor tissues (n=20), cell lines embedded in paraffin (n=5) and paired FFPE/blood samples (n=10 each).

Of the 20 FFPE tumor tissues included in the validation cohort, 11 (55%) were biopsies at diagnostic and 9 (45%) at the time of progression. Concordant results with previous hotspot *EGFR*, *KRAS* and *BRAF* mutation analysis were obtained in all cases (*Table 1*). In sample 15, NGS detected two previously unrecognized non-V600 *BRAF* mutations, p.G469A in exon 11 and a rare p.W604C mutation in exon 15; while in sample 10, corresponding to a patient progressing to EGFR-TKIs, a resistance *EGFR* p.G742S mutation in exon 18 was found. The three non-hotspot mutations were confirmed by Sanger sequencing. Finally, sample 4 harbored a 12 pb insertion in *ERBB2* gene (exon 20) that is not included in the GeneRead™ QIAact Lung DNA panel and was therefore not detected by NGS. Regarding CNV analysis, most copy number gains apparent by NGS were confirmed by FISH. They included *ERBB2* (progression, patient 9), *EGFR* (baseline, CRC patient 14) and *MET* gene (progression, patient 6) amplifications; but also concomitant CNVs in re-biopsies of five *EGFR* mutant patients at progression, *EGFR* and *ERBB2* in patients 8 and 10, and *EGFR* and *MET* in patient 12. The only discordant case was the patient 13, where *MET* amplification was not confirmed by FISH. Finally, in the five cell lines tested,

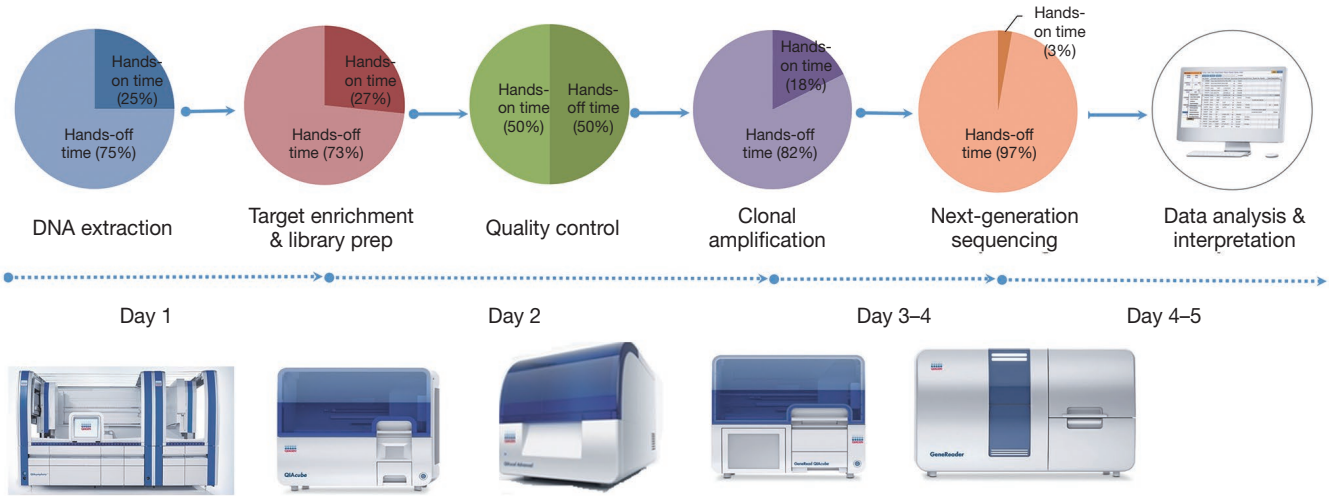

Figure 1 Workflow of GeneReader platform from cfDNA extraction to data analysis and interpretation. Hand-on times are represented in darker colors. At the bottom, the different integrated workflow equipment are illustrated in sequential order: QIAsymphony, QIAcube, QIAxcel, GeneRead QIAcube and GeneReader. For analysis and interpretation of raw data, two bioinformatic softwares (QCI-A and QCI-I) are used.

the results of the NGS analyses were fully concordant with the previous genotyping both for mutations and copy number variations. The only exception was the cell line NCI-H1781, which harbors a 3pb insertion in the exon 20 of *ERBB2* gene that is not included in the GeneRead^{TM} QIAact Lung DNA panel and therefore could not be detected by NGS.

Concordant results in NGS mutation analysis were obtained for all FFPE (n=10) and plasma (n=10) paired samples (*Table 2*). Single mutations in *EGFR* or *BRAF* genes were identified in six of these paired DNAs, while one pair harbored concomitant mutations in *EGFR* and *PIK3CA*. Finally, three samples pairs were wt for all genes in the NGS panel. The mutation allelic fractions (VAFs) determined by NGS in tissue were higher than the VAF in paired plasma samples, with the only exception of patient four. NGS also detected *EGFR* amplification in four *EGFR*-mutant FFPE samples. Of those, only patient four was also positive in paired plasma.

Prospective analysis of liquid biopsies and clinical characteristics of patients

From December 2017 to June 2018, we prospectively analyzed liquid biopsy samples from 24 advanced NSCLC patients progressing to different therapies, including 13 *EGFR*-mut patients treated with first and third generation EGFR-TKIs and nine EML4-ALK-positive patients relapsing to several ALK-TKIs. None of the 24 patients had a re-biopsy available for molecular analysis at the time to progression (*Table 3*). Of the 13 *EGFR*-mut patients, seven harbored exon 19 deletions at presentation, three exon 20 insertions and three the exon 21-p.L858R point mutation. Regarding the nine *ALK*-positive patients, the specific *ALK-EML4* variant at presentation had been determined in five and was unknown in four. Finally, two *BRAF*-mut patients progressing to chemotherapy and anti-PD-L1 treatment were included in the study.

At the time to progression, 9/24 patients (37.5%) had developed a limited number of metastatic extracranial oligometastases in lung (n=4), liver (n=2) or pleura (n=3), while 5/24 patients (20.8%) showed metastases exclusively in the central nervous system (CNS) and 4/24 patients (16.7%) presented a systemic progression. Finally, data about metastatic sites was unavailable in six cases. CSF was collected in all patients with CNS progression, with the only exception of patient 17, where lumbar puncture was not feasible and plasma was used instead. Similarly, PF was analyzed in 2 of the 3 patients with pleural progression. Interestingly, paired plasma and non-blood fluids (PF or CSF) were simultaneously collected in two patients (11 and 22).

Table 1 Mutational status by the GeneRead™ QIAact Lung DNA UMI and concordance with previous genotyping in the FFPE samples of the validation cohort

Sample	Cancer type	Type of sample	Region of pathogenic variants	Gene	Exon	Mutation (amino acid change)	VAF mutation (%)	Concordance (mutations) with previous genotyping	CNVs analysis	Concordance (CNVs) with previous genotyping
1	Lung	B	55259514..55259515	*EGFR*	21	p. L858R	15	Yes	No detected	Yes
2	Lung	B	25398284	*KRAS*	2	p. G12V	46.61	Yes	No detected	Yes
3	Lung	B	–	–	–	No pathogenic alterations	–	Yes	No detected	Yes
4	Ovary	B	–	–	–	No pathogenic alterations	–	Yes*	No detected	Yes
5	Lung	P	55242468..55242479	*EGFR*	19	p.L747_T751delinsP	21.32	Yes	*EGFR* amplification	Yes
6	Lung	P	55259515	*EGFR*	21	p. L858R	9.69	Yes	*MET* amplification	Yes
7	Lung	P	55259515	*EGFR*	21	p. L858R	4.65	Yes	No detected	Yes
8	Lung	P	55259515	*EGFR*	21	p. L858R	6.29	Yes	*EGFR* and *ERBB2* amplification	Yes
9	Lung	P	55242465..55242479	*EGFR*	19	p. E746_A750del	19.5	Yes	*ERBB2* amplification	Yes
10	Lung	P	55242467..55242485	*EGFR*	19	p. E746_S752delinsV	31.72	Yes	*EGFR* and *ERBB2* amplification	Yes
			55241722	*EGFR*	18	p. G742S	14.84			
11	Lung	P	55242465..55242479	*EGFR*	19	p. E746_A750del	10.74	Yes	No detected	Yes
12	Lung	P	55259515	*EGFR*	21	p. L858R	46	Yes	*EGFR* and *MET* amplification	Yes
13	Lung	P	55259515	*EGFR*	21	p. L858R	40.3	Yes	*EGFR* and *MET* amplification	No**
14	CRC	B	25398284	*KRAS*	2	p. G12V	47.08	Yes	*EGFR* amplification	Yes
15	Lung	B	140481402	*BRAF*	11	p. G469A	34.29	Yes	No detected	Yes
			140453123	*BRAF*	15	p. W604C	37.46			
16	Lung	B	55259515	**EGFR**	21	p. L858R	8.79	Yes	No detected	Yes
17	Lung	B	140453136	*BRAF*	15	p. V600E	31.65	Yes	No detected	Yes
18	Lung	B	–	–	–	No pathogenic alterations	–	Yes	No detected	Yes
19	Lung	B	25398281	*KRAS*	2	p. G13D	34.03	Yes	No detected	Yes
20	Lung	B	25398284	*KRAS*	2	p. G12D	31.2	Yes	No detected	Yes

Table 1 (*continued*)

Table 1 (*continued*)

Sample	Cancer type	Type of sample	Region of pathogenic variants	Gene	Exon	Mutation (amino acid change)	VAF mutation (%)	Concordance (mutations) with previous genotyping	CNVs analysis	Concordance (CNVs) with previous genotyping
NCI-H1975	Lung	CL	55259515	*EGFR*	21	p. L858R	78.3	Yes	No detected	Yes
			55249071	*EGFR*	20	p. T790M	76.3			
Hs 746T	Stomach	CL	116412044	*MET*	14	c.3082+1G>T (splicing variant)	99	Yes	*ERBB2* and *MET* amplification	Yes
NCI-H1781	Lung	CL	–	–	–	No pathogenic alterations	–	Yes*	No detected	Yes
EBC-1	Lung	CL	–	–	–	No pathogenic alterations	–	Yes	*MET* amplification	Yes
NCI-H596	Lung	CL	178936091	*PIK3CA*	10	p. E545K	52.3	Yes	*EGFR* amplification	Yes
			55249071	*MET*	14	c.3082+1G>T (splicing variant)	95			

*, patient 4 and cell line NCI-H1781 presents exon 20 ERBB2 insertions (p.G776>VC and p.Y772_A775dup respectively), not included in the panel; **, discordant result for *MET* amplification between NGS (positive) and FISH (negative). VAF, variant allelic fraction; CNV, copy number variation; B, baseline. P, progression. CL, cell line.

Genetic alterations in liquid biopsies at progression to targeted therapies

Liquid biopsies at progression were analyzed by NGS using the QIAact Lung DNA UMI Panel in order to identify genetic alterations associated with acquired resistance to targeted therapies. The results of the analysis are summarized in *Table 4*. Among the seven *EGFR*-mut patients progressing to first generation EGFR-TKIs, NGS detected the primary sensitizing mutation in 5 (71.4%). In contrast, the secondary p.T790M resistance mutation did not appear in any case, a result that was confirmed by PNA-Q-PCR (data not shown) (27). However, other genetic alterations potentially associated with the emergence of resistance to TKIs were detected, including *MET* amplification in one patient and a p.L833V mutation in exon 21 of the *EGFR* gene in patient 7. Re-analysis of the initial biopsy demonstrated that this mutation was not present at presentation (data not shown). Interestingly, the VAFs of the sensitizing p.L858R and the potentially resistant p.L833V mutations in the plasma sample at progression were very similar (0.84% and 0.89% respectively).

In the group of six patients progressing to osimertinib, the p.C797S resistance mutation appeared in 3 (50%), accompanied in all cases by the initial *EGFR* sensitizing mutation and the p.T790M. Visual inspection of the reads revealed that the p.C797S and the p.T790M were in *cis* configuration in all cases (*Figure 2*). In the remaining three patients, only the sensitizing mutation in *EGFR* could be detected. The high allelic fractions observed in two cases strongly suggest disappearance of the p.T790M as a mechanism of resistance. Regarding CNVs, concomitant *EGFR* and *MET* amplification was detected in two patients. Interestingly, paired plasma and pleural fluid samples were available for patient 11. The sensitizing *EGFR* exon 19 deletion was detected with greater reliability and higher VAF in plasma. This patient was undergoing a systemic progression disease with multiple metastatic sites besides pleura (*Figure 3*).

Among the 9 patients progressed to *ALK*-TKIs, mutations in exons 22, 23 or 25 of *ALK* associated with acquired resistance were detected in 5 (55.6%), while a *KRAS* p.G12V mutation emerged in one. Finally, *MET* amplification was not detected in any case, but *EGFR* amplifications were found in two patients, both harboring *ALK* resistance mutations. In Patient 17, no alterations were detected by NGS in the plasma analysis. The negative results could be due to the fact that, despite a CNS

Table 2 Mutational status by GeneReadTM QIAact Lung DNA UMI and concordance with previous genotyping in the paired FFPE/blood samples of the validation cohort

Sample	Cancer type	Type of sample	Region of pathogenic variants	Gene	Exon	Mutation (amino acid change)	VAF mutation (%) in FFPE	VAF mutation (%) in plasma	Concordance (mutations) with previous genotyping	CNVs analysis (FFPE)	CNVs analysis (plasma)
1	Lung	B	178952085	*PIK3CA*	21	p.H1047R	49,42	5.3	Yes	No detected	No detected
			55249010^55249011	*EGFR*	20	p. N771_H773dup	27,49	3.48			
2	Lung	B	140481402	*BRAF*	11	p. G469A	21.13	2.67	Yes	No detected	No detected
3	Lung	B	55242465..55242479	*EGFR*	19	p. E746_A750del	19.53	0.51	Yes	*EGFR* amplification	No detected
4	Lung	B	55249005^55249006	*EGFR*	20	p. V769_ D770insGTV	31.02	59.8	Yes	*EGFR* amplification	EGFR amplification
5	Lung	B	55249010^55249011	*EGFR*	20	p. N771_H773dup	59.65	4.0	Yes	*EGFR* amplification	No detected
6	Melanoma	B	140453136	*BRAF*	15	V600E	22.71	14	Yes	No detected	No detected
7	Lung	B	–	–	–	No pathogenic alterations	–	–	Yes	No detected	No detected
8	Lung	B	–	–	–	No pathogenic alterations	–	–	Yes	No detected	No detected
9	Lung	B	55259515	*EGFR*	21	p. L858R	46.15	7.4	Yes	*EGFR* amplification	No detected
10	Lung	B	–	–	–	No pathogenic alterations	–	–	Yes	No detected	No detected

VAF, variant allelic fraction; CNV, copy number variation; B, baseline.

Table 3 Clinical characteristics of patients with progressive disease included in the study

Characteristics	Total of patients (N=24)
Gender	
Male	10 (41.7%)
Female	14 (58.3%)
Smoking status	
Never smokers	14 (58.3%)
Former smokers	9 (37.5%)
Smokers	1 (4.2%)
Histology	
Adenocarcinoma	20 (83%)
NSCLC (NOS)	4 (17%)
Type of progression	
Extracranial oligometastases	9 (37.5%)
CNS progression	5 (20.8%)
Systemic progression	4 (16.7%)
Unknown	6 (25.0%)
Type of treatment	
First or second-generation EGFR TKI	7 (29.2%)
Third-generation EGFR TKI	6 (25.0%)
ALK inhibitors	9 (37.5%)
Other targeted therapies	2 (8.3%)

CNS, central nervous system; NSCLC, non-small cell lung cancer; NOS, not otherwise specified.

progression, lumbar puncture was not feasible and only plasma could be analyzed. Invalid results were obtained for patient 22, due to a low amount of purified cfDNA. This patient presented CNS progression, but the size of the metastases was very small and they were located in a limited area of the brain.

Finally, two patients (23 and 24) progressing to therapies other than TKIs were included in the study. Patient 23 presented the *BRAF* p.G469V at diagnostic, which reappeared in plasma with the progression of the disease. No other mutations or CNVs were observed. Patient 24 presented a *BRAF* mutation at diagnostic (p.V600E), which could be detected in ascites after systemic progression to immunotherapy, together with *de novo EGFR* amplification

Discussion

In this study, we present the results obtained in our hospital after the implementation of the GeneReader NGS platform for the routine analysis of liquid biopsies in NSCLC patients progressing to targeted therapies. Liquid biopsy samples are of particular relevance in this setting since, although a significant number of patients cannot be re-biopsied, genetic testing is recommended to select subsequent lines of treatment or to enroll patients in appropriate clinical trials (28). At this respect, several studies have demonstrated the utility of T790M analysis in blood to select patients for osimertinib, a procedure that permits to avoid unnecessary biopsies (29). In our case, re-biopsies could not be obtained in any case and liquid biopsies were the only samples available for testing.

Mechanisms of resistance *EGFR* and *ALK*-TKIs include secondary and "tertiary" mutations in the exons coding the tyrosine kinase domains of these receptors; amplification of *MET* or *ERBB2* and acquired mutations in *PIK3CA* or *KRAS* (30). All of these alterations can be detected by the NGS panel used in our study. Of the 21 patients progressing to TKIs with valid results in liquid biopsy, NGS analysis identified a potential mechanism of resistance in 12 (57%). Similarly to the literature, the most common acquired alterations detected in our study were secondary and "tertiary" mutations in *ALK* and *EGFR*, which appeared in 8/21 patients (38%), followed by amplifications in 5/21 patients (24%), and *KRAS* mutations in one patient (5%). Finally, we identified loss of the p.T790M in two patients progressing to osimertinib. Amplification of *EGFR* is frequently associated with *EGFR* sensitizing mutations baseline (31,32) and re-appears, together with them, in liquid biopsies after progression. Consequently, amplification of *MET* was considered a potential mechanism of resistance in all cases but *EGFR* copy gains only in patients progressing to *ALK*-TKIs, where *EGFR* pathway activation has been described to emerge only after relapse (33,34). Mechanisms of resistance have been described to co-occur in a significant percentage of patients progressing to targeted therapies. In our study, concomitant alterations associated with resistance could be identified in 3 of the 21 liquid biopsies (14%), namely a C797S mutation together with a *MET* amplification in a patient progressing to osimertinib, and secondary *ALK* mutations together with *EGFR* amplification in two patients relapsing to *ALK*-TKIs.

The T790M was not detected in any of the seven liquid biopsies corresponding to patients progressing to erlotinib

Table 4 Results of NGS analysis in liquid biopsies of patients progressing to targeted therapies

Progression to	Patient Nº	Type of sample	Treatment	Gene	Exon	Mutation (amino acid change)	VAF mutation (%)	CNVs analysis
Progression to first- or second-generation TKI	1	P	Erlotinib	*EGFR*	20	p.A767_V769dup	26%	*EGFR* amplification
	2	P	Erlotinib			No mutation detected		*EGFR* amplification
	3	P	Afatinib	*EGFR*	20	p.V769_D770insGTV	45.59%	*EGFR* amplification
	4	P	Erlotinib			No mutation detected		No CNVs detected
	5	PF	Gefitinib	*EGFR*	21	p.L858R	13%	*EGFR* and *MET* amplification
	6	PF	Erlotinib	*EGFR*	19	p.T751_E758delTSPKANKE	95%	*EGFR* amplification
	7	P	Erlotinib	*EGFR*	21	p.L858R + p.L833V[&]	0.89% + 0.84%[&]	No CNVs detected
Progression to third-generation TKI	8	CSF	Osimertinib	*EGFR*	19, 20	p.E746_A750del + p.T790M[&] + p.C797S[&] (*)	6.87% + 5.14%[&] + 0.65%[&]	No CNVs detected
	9	CSF	Osimertinib	*EGFR*	19, 20	p.E746_A750del + p.T790M[&] + p.C797S[&] (*)	16.69% + 16.53%[&] + 11.21%[&]	*EGFR* amplification
	10	P	Osimertinib	*EGFR*	19, 20	p.E746_A750del + p.T790M[&] + p.C797S[&] (*)	15.76% + 3.59%[&] + 4.77%[&]	*EGFR* and *MET* amplification
	11	P	Osimertinib	*EGFR*	19	p.T751_E758del	1.56%	No CNVs detected
		PF			19	p.T751_E758del (**)	<0.1%	
	12	P	Osimertinib	*EGFR*	21	p.L858R	26%	*EGFR* and *MET* amplification
	13	P	Osimertinib			No mutation detected		No CNVs detected
Progression to ALK inhibitors	14	CSF	Alectinib	*ALK* + *PIK3CA*	23 + 8	p.F1174L + p.F1174C + p.C420R[&]	3.27%[&] + 4.03%[&] + 7.59%[&]	No CNVs detected
	15	P	Crizotinib	*KRAS*	2	p.G12V[&]	0.81%[&]	No CNVs detected
	16	P	Brigatinib	*ALK* + *EGFR*	22 + 19	p.I1171N + p.P741L[&]	3.17% + 8.10%	*EGFR* amplification
	17	P	Crizotinib			No mutation detected		No CNVs detected
	18	P	Crizotinib			No mutation detected		No CNVs detected
	19	P	Lorlatinib			No mutation detected		No CNVs detected
	20	P	Alectinib	*ALK*	25	p.G1269A[&]	0.5%[&]	No CNVs detected
	21	P	Brigatinib	*ALK*	23	p.F1174L[&]	1.38%[&]	*EGFR* amplification
	22	P	Brigatinib			Invalid results		No CNVs evaluable
		CSF				Invalid results		No CNVs evaluable
Progression to other targeted therapies	23	P	Carboplatin-pemetrexed	*BRAF*	11	p.G469V	2.87%	No CNVs detected
	24	AF	Ipilimumab-nivolumab	*BRAF*	15	p.V600E	19.34%	*EGFR* amplification

[&], mutations associated with resistance; *, patients 8, 9 and 10: p.T790M and p.C797S resistance mutations were observed in CIS configuration (for more details see *Figure 2*); **, patient 11: the p.T751_E758del in exon 19 was observed in PF only with manual inspection (for more details see *Figure 3*). PF, pleural fluid; CSF, cerebrospinal fluid; AF, ascites; VAF, variant allelic fraction; CNV, copy number variations.

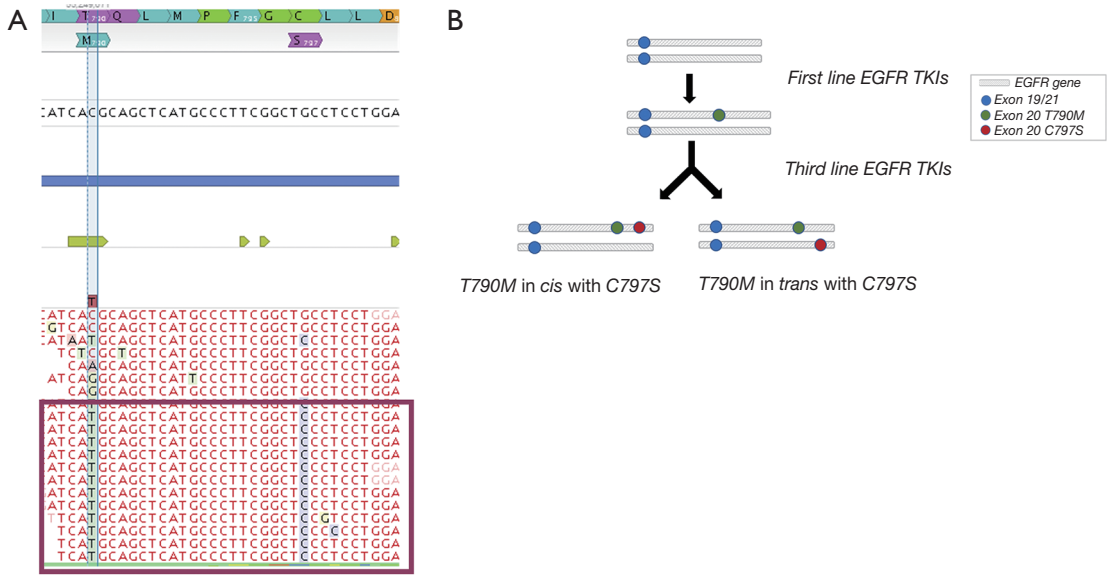

Figure 2 Acquired resistance to osimertinib: (A) QCI Analyze (QCI-A) results of patient 9 showing the alignment of reads (based on Genome Reference Consortium hg19) at the variant positions p.T790M and p.C797C of *EGFR*, together with the induced amino acid change; (B) dynamic representation of the emergence of resistance mutations in patients treated with *EGFR* TKIs. NGS sequencing is required for the assessment of the allelic configuration of the C797S mutation. If the C797S appears in *trans* with the T790M, tumor cells are potentially sensitive to the combination of a first-generation and a third-generation *EGFR* TKI. If the C797S is in *cis* with the T790M, tumor cells are resistant to all *EGFR*-TKIs.

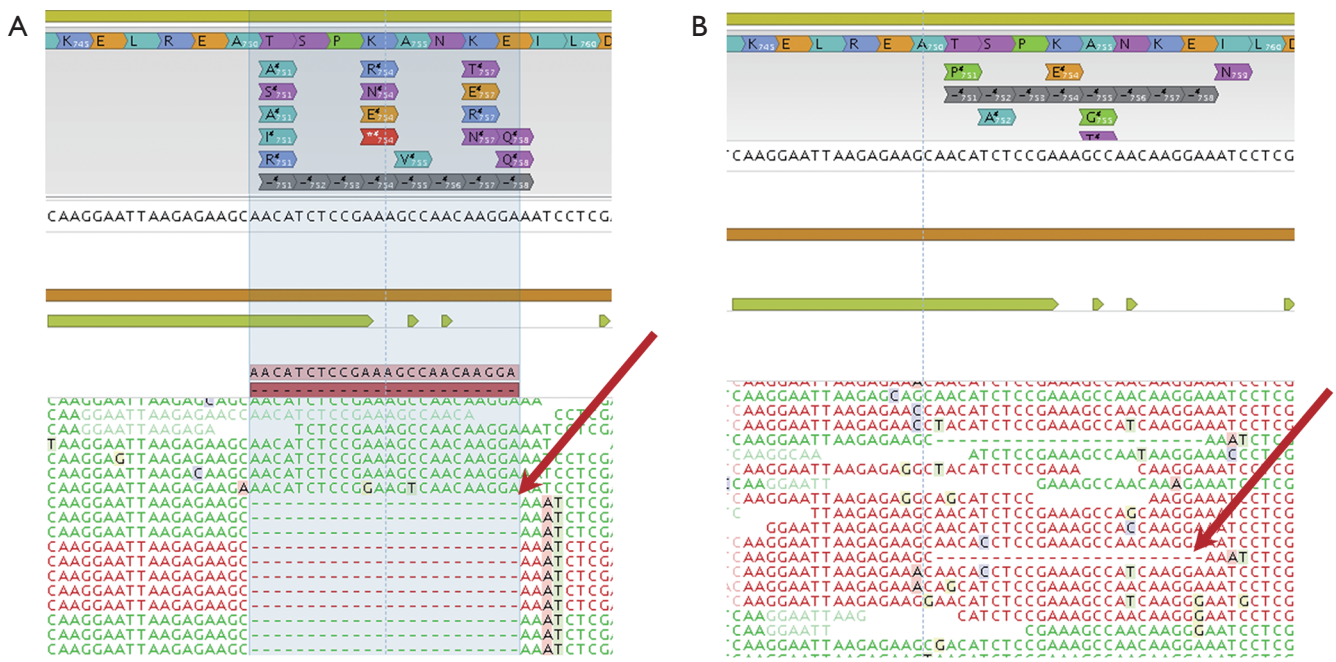

Figure 3 QCI Analyze (QCI-A) results of patient 11 showing the alignment of the reads at the variant position p.T751_E758del of *EGFR*. (A) Results in blood; (B) results in pleural fluid. The dotted gaps correspond to the presence of exon 19 p.T751_E758del.

and gefitinib. In two cases, the initial *EGFR* sensitizing mutation did not appear either and the presence of the T790M in tumor tissue could not be ruled out. In one patient relapsing to gefitinib, NGS detected a rare mutation in exon 21 of *EGFR* (p.L833V), which has been associated with resistance to *EGFR*-TKIs (35). In the case of patients progressing to osimertinib, we found the "tertiary" resistance mutation C797S in three of six liquid biopsies (50%). The mutation was in "*cis*" configuration with the T790M in all cases. Only NGS platforms can differentiate the "*cis*" *vs.* "*trans*" configuration, which is clinically relevant in order to determine whether the patient can be re-challenged with *EGFR*-TKIs (8,36,37). Loss of T790M was identified in two additional cases of patients in progression to osimertinib, where the *EGFR* sensitizing mutations reappeared at allelic fractions >1% but the T790M was not detected. Finally, mutations in the exons coding for the tyrosine kinase domain of *ALK* were identified in 4 of 8 patients (50%) progressing to *ALK*-TKIs. One of them presented simultaneously with two *ALK* resistance mutations and a *PIK3CA* mutation (p.C420R) that has been described as oncogenic in cell models (38), and another showed the p.I1171N resistance mutation in *ALK* and a mutation of uncertain significance in the exon 19 of *EGFR* (p.P741L). Unfortunately, samples at presentation were not available to determine if these mutations in *PIK3CA* and *EGFR* were also associated with acquisition or resistance. Finally, a p.G12V mutation in *KRAS* was found in a patient progressing to crizotinib. This particular mutation has already been reported as a mechanism of acquired resistance in translocated *ALK* patients treated with this drug (39,40).

The published studies on genetic testing by NGS in liquid biopsies have been generally limited to cfDNA isolated from plasma. In contrast, we have included eight fluids other than blood in our report. Four of them were cerebrospinal fluids of patients with CNS progressions; three were pleural fluids and one ascites. Remarkably, with the only exception of one sample with invalid results, we could detect genetic alterations in all of them, namely sensitizing mutations, resistance mutations and/or copy number alterations. The ascites sample was from a patient with rapid progression to immunotherapy and showed *EGFR* amplification, a genetic aberration that has been associated with hyperprogression to anti-PD-1/PD-L1 agents (41). Taken together, our results suggest that, depending on the site of progression, fluids other than blood can be used for cfDNA purification and subsequent NGS analysis.

Our study also had some limitations, some of them inherent to NGS techniques. First, some mechanisms associated with resistance could not be detected, such as EMT or SCLC transformation. Second, as discussed above, we found some mutations of uncertain significance. Third, due to the lack of paired tumor biopsies, the amplifications detected by NGS could not be validated by the gold standard technique, FISH. However, the percentage of liquid biopsies baseline positive for copy number gains by NGS (*Table 2*) was only 10%, compared to 24% after progression; suggesting that the amplifications determined by NGS were not false positives.

In summary, our study demonstrates that NGS can be implemented in routine clinical practice for the genetic analysis of liquid biopsy samples in patients progressing to targeted therapies. NGS can detect most of mechanisms associated with acquired resistance and provide useful information of the selection of second and subsequent lines of treatment.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Studies were conducted in accordance with the Declaration of Helsinki (as revised in 2013) under an approved protocol of the institutional review board of the Quirón Hospitals, and de-identified for patient confidentiality. Informed written

consent was obtained from all subjects.

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