Clinical evaluation of the utility of a liquid biopsy (Circulating tumoral cells and ctDNA) to determine the mutational profile (EGFR, KRAS, ALK, ROS1 and BRAF) in advanced NSCLC patients.

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ABSTRACT

Background
Circulating tumor DNA (ctDNA) has emerged as a specific and sensitive blood-based source for detection of several mutations in non–small cell lung cancer (NSCLC). Other clinical applications for ctDNA include molecular assessment of patients at diagnosis and serial (realtime) monitoring of biomarker status or the development of resistance mutations.

Methods
Sixty patients with advanced NSCLC who either (1) had a new diagnosis or (2) developed acquired resistance to an EGFR kinase inhibitor were analyzed with highly sensitive Biocept, Inc Target Selector™ qPCR based plasma genotyping tests for detection of the EGFR mutations L858R, Del19 and T790M. In addition, group 1 was analyzed for KRAS, BRAF, ROS1 and ALK and circulating tumor cells (CTCs) before and after TKI treatment.

Results
Our results showed concordance rates of EGFR, KRAS and ALK mutations of up to 90% between the tissue and blood samples. The T790M mutation analyzed in plasma of patients with clinical progression to TKI inhibitors was found in 50% of the cases. Group 1 paired analysis of mutations status monitoring (P= 0.016) showed that the pattern of mutant ctDNA and CTCs changed in response to systemic therapy in 83% of the cases (Partial response or disease progression; R2=0.808). ctDNA analysis of multiple mutations showed that 40% of patients had at least one additional mutation not detected by the tissue biopsy; 28% of EGFR tissue positive patients also had a KRAS mutation. Furthermore, 75% of KRAS positive patients had a BRAF mutation. These results demonstrate that ctDNA analysis may frequently detect mutations missed by standard tissue genotyping due to tissue heterogeneity.

Conclusions
Target Selector™ ctDNA assays are capable of rapidly detecting EGFR, KRAS and BRAF mutations with the robustness needed for real world testing. It is highly concordant with mutations present in tumor tissue and therefore appears to be a viable noninvasive alternative to identifying resistance EGFR mutations such as T790M in patients who progress on first line TKI therapy as well as for realtime monitoring of patients' clinical status. As third generation EGFR T790M inhibitors come into clinical use, the need for rebiopsy and potential role of plasma genotyping will expand dramatically.

STUDY DESIGN

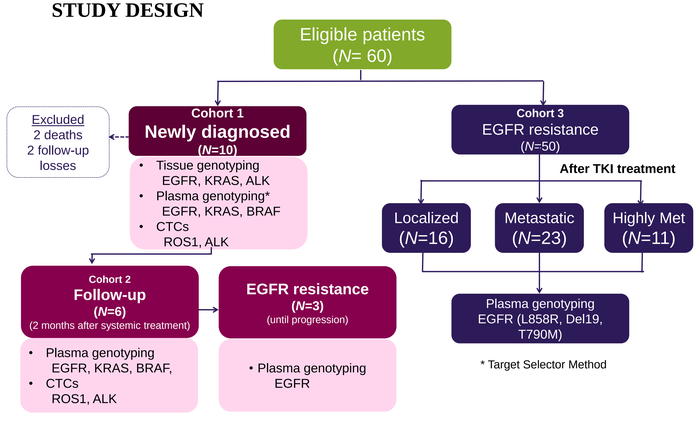

Figure 1. Study Design. 60 patients with advanced NSCLC were consented to a prospective study of Target Selector based plasma genotyping and CTC analysis. 10 patients were newly diagnosed (cohort 1) and 6 of them underwent serial plasma genotyping and CTC analysis (cohort 2). 50 patients had acquired resistance to EGFR kinase inhibitors (cohort 3). Plasma genotyping and CTC analysis, together with tissue genotyping was successfully completed for 10 patients for EGFR exon 19/L858R, T790M, KRAS, ALK. 4 Patients were excluded from analysis (cohort 1) if they did not complete the blood draw or tissue genotyping failed or was not performed. BRAF and ROS1 was completed using plasma genotyping and CTC testing for 6 patients (cohort 2). 50 patients (cohort 3) were completed for plasma genotyping for EGFR exon 19/L858R, T790M.

METHODS

Patients

We recruited a combination of newly diagnosed individuals, patients demonstrating TKIs resistance, and patients with proven stage IV NSCLC under medical care in the Clinical Research Division of the National Institute of Cancer, Mexico City. Patients with small-cell lung cancer, that were Eastern Cooperative Oncology Group (ECOG) performance status 3, or who could not give informed consent, were excluded from recruitment. The study was approved by the National Institute of Cancer Authority Institutional Review Board and Ethics Committee. Tumor biopsy samples were analyzed using routine histopathological and molecular pathology procedures on formalin-fixed, paraffin-embedded specimens for EGFR, KRAS and ALK patients (Cohort 1 and 2) and for EGFR mutations (Cohort 3). Recruited patients were followed up for their subsequent management for treatment response, disease control and progression, lines of treatment, and duration of progression-free survival (PFS) and overall survival (OS).

Venous blood samples (10-20 mL) were collected from all subjects before treatment on the day of starting anticancer therapy, and at the time of progression. The choice of treatment was determined by the attending physicians without knowing the EGFR mutation plasma status. At recruitment, subject demographic characteristics were recorded. The recruited subjects were followed up at 3-4 week intervals. Chest x-rays were performed at every visit to check for signs of clinical progression and if in doubt, computed tomography (CT) or positron emission tomography-CT was performed. PFS was taken as the duration from the commencement of first-line anticancer therapy to the day disease progression was first found using chest imaging or physical examination that showed new sites of involvement like regional lymph node enlargement. OS was taken from the commencement of first-line anticancer therapy to either the day of death or last clinic follow-up. Blood samples were collected in Biocept CEE-SURE™ tubes, and sent for analysis to Biocept (San Diego, CA).

Epidermal Growth Factor Receptor Exon 19 Deletion / Exon 21 L858R / Exon 20 T790M, KRAS, and BRAF in Plasma; ALK and ROS1 in CTCs

Four 8-10 ml tubes of blood were collected from newly diagnosed patients and patients progressing on TKI treatments. Patient samples were then tested for EGFR, KRAS, BRAF, (Cohorts 1 and 2) or for EGFR (L858R, DEL19 and T790M) (Cohort 3) using Biocept's minimally invasive Target-Selector™ assay for ROS and ALK using CTC FISH assays. The Target-Selector™ assay combines real-time PCR and sequencing to verify the presence of mutations. Its unique design blocks wild-type amplification, allowing rare mutant sequences to be amplified in a high prevalence of the wild-type allele. Results from these "liquid-biopsies" were compared to results obtained on standard tissue biopsy. Sensitivity, concordance and positive predictive value (PPV) were calculated.

Circulating nucleic acid was extracted from blood plasma and used in Target-Selector™ assays specific for the amplification of BRAF, KRAS, or EGFR kinase mutations: (1) with deletion 746-750 in exon 19, (2) the L858R point mutation within exon 21, and (3) T790M within exon 20. Target-Selector™ uses forward and reverse primers as well as a Target-Selector™ probe to specifically block wild-type amplification and allow enrichment of mutant sequences. The Target-Selector™ probe serves as a wild-type blocker as well as a detection probe for amplification. Sanger sequencing of the amplified Target-Selector™ product is used to confirm presence of the mutations mentioned. Exon 19 deletion and L858R are the two most common EGFR activating mutations seen in patients with NSCLC, whereas T790M is associated with acquired resistance to TKI therapy.

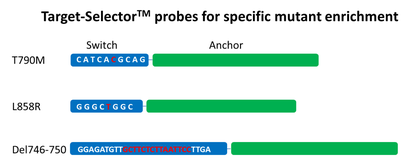

Figure 2. Target-Selector™ probes consist of two domains: a switch domain which spans the mutation region of interest (marked in red for the individual mutations) and an anchor domain which allows efficient hybridization to the target region. They also contain quencher and fluorescent label for detection of amplified product (not shown).

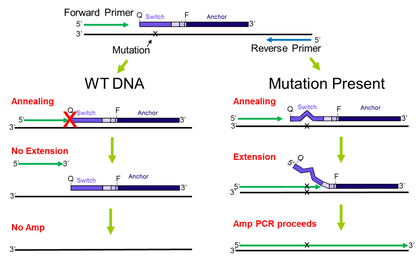

Figure 3. Target-Selector™ probes block wild-type amplification but allow mutant amplification. When a mutation is found in the DNA template this leads to a reduction of the melting temperature of the switch portion of the Target-Selector™ probe for the mutant DNA template, which in turn allows the forward primer under the PCR cycling condition to extend through the switch portion. In the case of the wild-type DNA template the forward primer cannot extend through the switch portion. Once the temperature is increased during PCR cycling, the forward primer in the case of the wild-type falls off the wild-type DNA template without extending, but can complete extension through the mutant DNA template.
The Target-Selector™ probe allows the mutant to efficiently amplify in PCR reactions over a wide extension temperature window compared to the wild-type template which is efficiently blocked

RESULTS

A total of 60 patients with advanced NSCLC were enrolled in the study with either newly diagnosed disease (N = 10) or acquired resistance to an EGFR TKI (N = 50). All patients except one, had adenocarcinoma histologic subtype (not shown), and all of them had stage IV disease (Table 1). Patients were predominantly female (46/60). Patients who did not complete their initial blood sampling or any tissue genotyping were excluded from the analysis (Figure 1).

Table 1. Baseline characteristics of NSCLC patients

Characteristics	Total	Cohort 1 % (N) Newly Diagnosed	Cohort 2 % (N) Acquired Resistence
Sex %(N)			
Male	23.3 (14/60)	40 (4/10)	20 (10/50)
Female	76.7 (46/60)	60 (6/10)	80 (40/50)
Age (years)			
Median (Range)	63 (38 - 90)	70 (44 - 79)	62.5 (38 - 90)
Clinical State at Dx			
IIIB	0 0/60)	0 (0/10)	0 (0/50)
IV	100 (60/60)	100 (10/10)	100 (50/50)
Type of Disease			
Localized	30 (18/60)	20 (2/10)	32 (16/50)
Metastatic	46.7 (28/60)	50 (5/10)	46 (23/50)
Highly metastatic	23.3 (14/60)	30 (3/10)	22 (11/50)
CNS metastases			
Positive	51.7 (31/60)	30 (3/10)	56 (28/50)
Negative	48.3 (29/60)	70 (7/10)	44 (22/50)
Treatment Lines			
≤ 3	65 (39/60)	100 (10/10)	58 (29/50)
≥ 4	35 (21/60)	0 (0/10)	42 (21/50)

Abbreviations: NSCLC= Non-Small Cell Lung Cancer, CNS mets=Central Nervous System metastases.

Table 2. Biopsy EGFR / KRAS / ALK mutant (N=6)

Patient	Cohort 1 Biopsy mutation detected	Biopsy mutation not detected	Alteration(s) detected in blood	Mutation(s) not detected in blood
2	DEL19		DEL19	
3	DEL19	L858R KRAS (G12C)	DEL19 + L858R KRAS (G12C)	
4	L858R	KRAS (G12C)	L858R KRAS (G12C)	
6	DEL19	L858R	DEL19 + L858R	
7	(ND)		(ND)	
9	DEL19	EML4-ALK	EML4-ALK	DEL19

Abbreviations: ND= No mutation detected

Table 3. Diagnostic Sensitivity and Specificity, and Positive and Negative Predictive Values of Target Selector™ Method of EGFR mutation detection in Plasma

	Estimated Value (%)	95% CI Lower Limit	95% CI Upper Limit
Sensitivity	90	82.83	95.10
Specificity	100	96.38	100
Positive Predictive Value	100	95.98	100
Negative Predictive Value	90.91	83.92	95.55

Plasma Target Selector™ detection exhibited high specificity for the detection of EGFR activator mutations (DEL19 and L858R) (100% [7 of 7]), and KRAS G12C (100% [1 of 1]). Positive predictive value was also high for assays at 100% (Table 2). Overall assay sensitivity was of 90%.

Table 4. Follow-up newly diagnosed patients two months after initial treatment (n=6)

Patient	Blood Genotyping: Tissue Genotyping	Mutations before treatment	Mutations after treatment	Clinical Outcome: Treatment	Response
2	DEL19	DEL19	None	Gefitinib	Partial Response RECIST:36%
3	DEL19	DEL19, L858R, KRAS (G12C)	None	Gefitinib	Disease Progression - CNS mets
4	L858R	L858R, KRAS (G12C), BRAF (V600E)	L858R	Erlotinib	Disease Progression - Carcinomatosis meningeal
6	DEL19	DEL19, L858R	None	Gefitinib	Partial Response RECIST: 68.7%
7	None	None	None	Carboplatin + Pemetrexed	Stable Disease - Increased metabolic rate
9	DEL19	ALK	DEL19	Gefitinib	Partial Response RECIST: 40%

Table 5. Evaluating Progressive Disease and its impact on T790M detection

Mutation	Localized Disease % (N)	Metastatic Disease % (N)	Highly Metastatic Disease % (N)
Negative	44.4 (8/18)	60.7 (17/28)	14.3 (2/14)
DEL 19	11.1 (2/18)	3.6 (1/28)	7.1 (1/14)
L858R	22.2 (4/18)	21.4 (6/28)	7.1 (1/14)
T790M	5.6 (1/18)	0 (0/28)	21.4 (3/14)
DEL 19 + L858R	5.6 (1/18)	3.6 (1/28)	0 (0/14)
DEL 19 + T790M	11.1 (2/18)	10.7 (3/28)	21.4 (3/14)
L858R + T790M	0 (0/18)	0 (0/28)	28.6 (4/14)

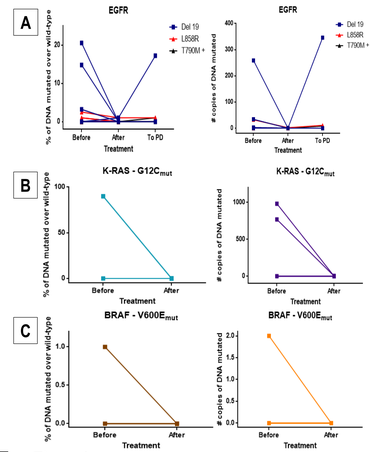

Figure 4. Paired samples analysis of, EGFR (A), KRAS (B) and BRAF (C) before and after treatment. Different patterns of changes in plasma mutation status were observed by Target Selector™.

T790M mutation was evaluated on 50 patients demonstrating TKI resistance. Fourteen patients had highly metastatic disease and 71.4% of them displayed the resistance mutation. In contrast, only 14.3% of metastatic patients and 22.3% of patients with localized disease harbored the T790M mutation. This may be due to the characteristics of the tumor, as extra-thoracic disease displays higher mutation levels that may be more easily detected in blood.

CONCLUSIONS

- Target Selector ™ ctDNA assay is capable of rapidly detecting EGFR, KRAS, and BRAF mutations with the robustness needed for real world testing. CTC testing can identify ALK and ROS1 translocations.
- This technique is highly concordant with mutations present in tumor tissue and therefore appears to be a viable noninvasive alternative to identify secondary EGFR mutations such as T790M in patients who progress on first line TKI therapy.
- Serial quantitative plasma Target Selector™ among patients revealed clear changes in the level of detectable mutant allele frequency after treatment.
- The use of this technology to monitor disease status in real time has potential utility for routine clinical care.
- Further studies are necessary to confirm that if T790M mutation detection depends on the tumor charge present in extra or intra thoracic disease.