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Article *in* Oncology · April 2021

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Received: October 13, 2020
Accepted: January 21, 2021
Published online: April 26, 2021

Cell-Free Circulating Tumor DNA Improves Standard Genotyping of Non-Small-Cell Lung Cancer and Increases Detection of Targetable Alterations in a Selected Hispanic Cohort

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Keywords
Non-small-cell lung cancer · Liquid biopsy · Cell-free DNA · Next-generation sequencing · Oncogenic driver · Gene mutations

Abstract

Background: Several studies have shown that the non-small-cell lung cancer (NSCLC) genomic background among Hispanics differs from other populations. The finding of low-frequency genomic alterations in cell-free DNA (cfDNA) can increase diagnostic accuracy and could improve treatment in NSCLC. ***Methods:*** Data from 54 Hispanic patients with advanced NSCLC with high clinical suspicion for *ALK*, *EGFR*, and *ROS1* mutations were collected (including young age, female sex, and non-smokers). cfDNA was extracted from plasma and analyzed using a commercial next-generation sequencing test (Guardant360) which detects genomic alterations in 74 genes. ***Results:*** The median age was 56 years (range 31–83). Most patients were female (661.1%) and never smokers (72.3%). Among the patients included, 96% (52/54) had cfDNA detectable alterations with a mean number of 3.37 cfDNA alterations per test (range 1–10). cfDNA was able to detect some genomic alterations previously undetected by tissue biopsy. Among patients with insufficient or unavailable tissue to perform testing, mutations in *EGFR* and *ALK* which led to a change in therapy were determined using cfDNA in 28.8 and 3.8% of cases, respectively. Among patients with cfDNA alterations, 46.1% ($n = 24$) were switched to a targeted therapy with a median progression-free survival of 11.1 months (95% CI 7.6–14.6) and an overall survival of 40.3 months (95% CI 27.1–53.6). Concurrent genetic mutations with *TP53* and *KRAS* negatively impacted the prognosis. ***Conclusions:*** In a selected population of NSCLC Hispanic patients, comprehensive cfDNA analysis allowed a treatment change in 46.1% of the cases. Guardant360 allows the identification of genomic alterations to improve treatment selection and increase prognosis.

Introduction

Non-small-cell lung cancer (NSCLC) most frequently presents at an advanced stage, when treatment options lack long-term efficacy. Biomarker-directed therapy has greatly transformed the approach to treating advanced NSCLC. Targeted therapies directed against receptor ty-

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rosine kinases (TKIs) lead to dramatic responses in subsets of patients with lung adenocarcinomas harboring activating genomic alterations in the corresponding kinase genes, including mutations in epidermal growth factor receptor (*EGFR*), and rearrangements in the anaplastic lymphoma kinase (*ALK*) and *ROS1* genes [1, 2]. Despite dramatic responses in some patients, the vast majority of patients treated with TKIs develop resistance to these drugs and have disease progression after 8–14 months following treatment initiation [3, 4]. We have previously reported the prevalence of mutations in *EGFR* and rearrangements in *ALK* in the Latin American population [5]. However, information on the occurrence of concurrent genetic alterations in advanced-stage NSCLC with a primary oncogenic driver is limited in this population, since most of the research in this regard has been conducted in populations comprised of non-Hispanic whites [6, 7]. Nonetheless, co-occurring genomic alterations can profoundly impact clinical outcomes, and therefore their occurrence should be studied in order to adequately sequence therapeutic strategies in patients with diverse ancestries [8, 9].

Tissue biopsy continues to be the standard of care for diagnosis and molecular characterization in NSCLC patients. However, tissue biopsy has several limitations, including the difficulty in acquiring adequate amounts of tissue, limited repeatability due to a suboptimal clinical condition or an unfavorable tumor site such as bone, the central nervous system, or multiple small pulmonary nodules. Furthermore, tissue biopsy has risks for major complications, representative samples are challenged by tumor heterogeneity and, lastly, cost issues should be taken into account, considering that a tissue biopsy is considerably more expensive than a blood draw [6, 7]. In recent years, a variety of liquid biopsy platforms have been developed that can serve as a complement to routine tissue-based diagnostics and, pivotally, as a feasible means of identifying acquired resistance mechanisms. Currently, liquid biopsy, especially the analysis of circulating tumor DNA (ctDNA) is recommended by several guidelines, including the CAP/IASLC/AMP guideline for molecular testing in patients with NSCLC [8].

Massive parallel (or next-generation) sequencing (NGS) allows detection of numerous genomic alterations in tens to hundreds of genes simultaneously, as well as detection of rare somatic mutations, and can be performed with liquid biopsy. Most NGS methods are based on polymerase chain reaction (PCR) amplification of platform-specific DNA fragment libraries, which are then sequenced. These techniques are well suited to high-throughput sequencing and are able to detect the full spectrum of genomic changes present in cancer. Different NGS-based methods have been developed and subsequently validated for NSCLC ctDNA mutation detection [9–12]. The Centers for Medicare and Medicaid Services (CMS) recently published a note supporting the use of NGS in the daily clinical practice cfMaMS [13]. Guardant360 is an NGS test for cell-free DNA (cfDNA) that detects all four major variant classes (point mutation or single nucleotide variants, SNVs; small insertions/deletions, indels; copy number amplifications, CNAs, and genomic rearrangements, SVs) in 74 cancer-related genes. This ctDNA assay has a sensitivity and specificity of >85 and >99.99%, respectively, for the detection of single-nucleotide variants in tumor of advanced cancer patients [10]. In the present study, we perform molecular analyses using Guardant360 in patients with both tissue biopsies and blood samples available.

Methods

Study Design and Patients

This was a prospective, descriptive, observational study conducted in 2 centers in Mexico and Colombia to detect genetic alterations in cfDNA using the Guardant360 test. We aimed to evaluate the clinical utility of liquid biopsy in detecting targetable alterations in a population of Hispanic patients with advanced lung adenocarcinoma.

The inclusion criteria were Hispanic patients with stage IIIB/IV lung adenocarcinoma according to the seventh edition of the American Joint Committee on Cancer staging, age >18 years, a World Health Organization (WHO) performance status of 0-2, unsuitable for curative treatment, irrespective of systemic treatment received previously. All patients had radiographic evidence of disease.

Samples underwent tissue screening for *ALK* and *EGFR*. Tissue samples must have had >10% of the tissue area in a formalin-fixed paraffin-embedded tissue biopsy occupied by invasive cancer cells as evaluated by a pathologist. *EGFR* mutations (exons 18–21) from samples were detected by a Therascreen RGQ PCR kit (QUIAGEN, Mexico City, Mexico; Scorpions ARMS method), according to the manufacturer's instructions. *ALK* rearrangement was detected with immunohistochemical assay or fluorescence in situ hybridization assay. Furthermore, 10-mL tubes of whole blood for plasma extraction were collected per individual in Streck Cell-Free DNA Blood Collection (Streck) tubes, which contain a proprietary formaldehyde-free preservative that stabilizes white blood cells, preventing the release of genomic DNA and allowing isolation of high-quality cfDNA [14]. Samples were sent to Guardant Health Inc. (http://www.guardanthealth.com) laboratories according to national and international biologic sample transportation standards. The analytic and clinical validation of the Guardant360 assay was described previously [15]..

Radiographic assessment was performed in accordance with international guidelines. The disease control rate was defined as a complete response (CR), a partial response (PR), or stable disease (SD), according with RECIST criteria v.1.1. The therapeutically

Table 1. Characteristics of the cohort (n = 54)

Sex	
Male	21 (38.8)
Female	33 (61.1)
Age, years	56 (31–83)
Performance status	
0–1	51 (94.4)
>1	3 (5.6)
Smoking history	
Yes	15 (27.7)
No	39 (72.3)
Extent of disease	
Locally advanced	2 (3.7)
Metastatic	52 (96.3)
Documented *EGFR* mutation (exon 19 and L858R)	
Yes	19 (35.2)
No	35 (64.8)
Documented *ALK/EML-4* gene fusion	
Yes	1 (1.9)
No	53 (98.1)
Line of therapy at the time of cfDNA molecular status determination	
First line	30 (55.6)
Second line	13 (24.1)
Third line	11 (20.4)

Data are presented as n (%) or the mean (min.–max.).

Table 2. Alterations in cfDNA (n = 52)

Mean number (min.–max.)	3.37 (1–10)
Patients with therapeutic switch (n = 24/52)	
EGFR mutation (exon 19 and L858R)	15 (28.8)
ALK/EML-4 gene fusion	2 (3.8)
HER2 (S280F and Exon20inser)	3 (5.8)
MET	1 (1.9)
BRAF	1 (1.9)
ROS1	1 (1.9)
RET	1 (1.9)
Concurrent genetic alterations in EGFR or ALK positive (n = 10)	
EGFR-TP53	7 (70)
EGFR-KRAS G13D	2 (20)
ALK/EML4-TP53	1 (10)
Response in patients who were switched to receive therapy according to the alterations detected by cfDNA (n = 22)	
Disease control rate	85.7
PR	60.7
SD	25
CR	0
Progressive disease	14.2

Data are presented as the mean (min.–max.), n (%), or %. PR, partial response; SD, stable disease; CR, complete response.

targetable driver mutations considered are summarized in the online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000514648).

Statistical Analysis

For descriptive purposes, continuous variables are summarized as arithmetic means with standard deviations or medians with interquartile ranges. Meanwhile, categorical variables consisted of frequencies and percentages. Comparisons were made using the one-way ANOVA or the Mann-Whitney U test, according to the data distribution as determined by the Kolmogorov-Smirnov test. The χ^2 test or Fisher's exact test were used for assessing the statistical significance of categorical variables, determined as p values <0.05 when using a 2-tailed test. Progression-free survival (PFS) was defined as the time from treatment start until radiographical documented progression using the RECIST criteria v.1.1 or loss to follow-up. Overall survival (OS) was defined as the time between diagnosis and death by any cause.

Results

Baseline Demographic Characteristics

A total of 56 patients with advanced lung adenocarcinoma were screened for enrollment in the study from January 2016 to January 2018. The median age was 56 years (range 31–83). Most patients were female (61.1%). The majority of patients had a WHO performance status of 0-1 (94.4%), and most were never smokers (72.3%). The patient demographics and clinical characteristics are summarized in Table 1. The median duration of follow-up was 19.3 months. Two patients did not complete their initial blood draw and were excluded from the analysis, therefore the final sample included 54 patients.

Frequently Identified Genetic Alterations

Genetic alterations in cfDNA were determined in patients undergoing their first (55.6%; n = 30), second (24.1%; n = 13), and third/further (20.4%; n = 11) line of treatment with systemic therapy. In 94% (n = 52), testing identified detectable cfDNA, with a mean number of 3.37 cfDNA alterations per test (range 1–10). Identified genetic alterations among patients in this study included mutations in *EGFR* (n = 19/52; 36.5%), Tp53 (n = 15/52; 28.8%), *ALK-EML4* fusion (n = 4/52; 7.6%), *KRAS* (n = 4/52; 7.6%), *BRAF V600E* (n = 4/52; 7.6%), *HER2* (n = 3/52; 5.7%), *CDKN2A* (n = 2/52; 3.8%), *RET* fusion (n = 1/52; 1.9%), *ROS1* (n = 1/52; 1.9%), and *MET* (n = 1; 1.9%). Among the 52 patients with cfDNA genomic alterations, nineteen had a previously known oncogenic driver in *EGFR* 36.5% (n = 19) and 1.9% a previously

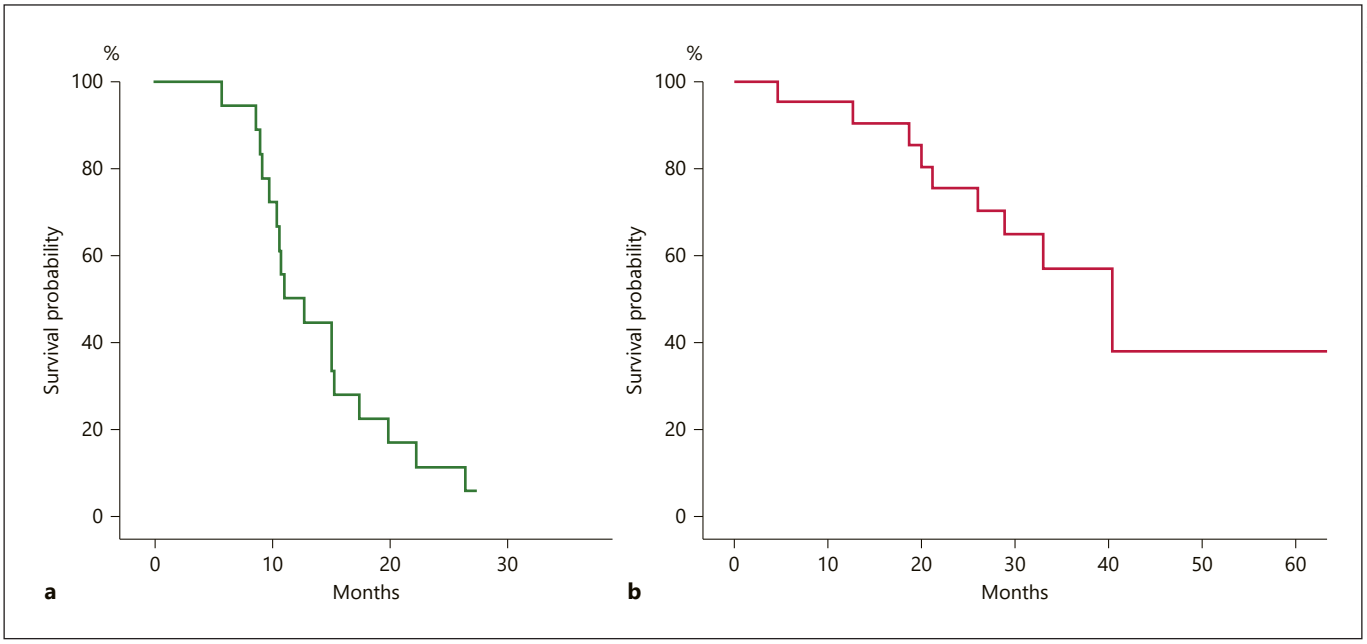


Fig. 1. PFS (**a**) and OS (**b**) for patients who switched therapy.

identified *ALK* fusion ($n = 1$). Among the 52 patients with identifiable cfDNA alterations, 46.1% ($n = 24$) were switched to a targeted therapy as a result of alterations detected through cfDNA which were previously undetected (or not tested) by tissue biopsy (Table 2).

Response and Survival Outcomes for Patients Who Switched Therapy according to cfDNA Results

After therapy switch, the disease control rate was 85.7%; PR was 60.7% and SD 25%. No CR was observed, while PD was observed in 14.2% (online suppl. Fig. 1). Median PFS for patients treated with TKIs was 11.1 months (95% CI 7.6–14.6). Furthermore, the median OS in patients who switched therapy after the detection of targetable alterations was 40.3 months (95% CI 27.1–53.6; Fig. 1). Patients who did not switch therapy following cfDNA identification of targetable mutations had a median OS of 22.3 months (95% CI 8.3–36.5; $p = 0.14$).

Concomitant Genetic Alterations in EGFR or ALK Positive NSCLC

Among *EGFR* and *EML4-ALK* NSCLC-positive patients, a concomitant genetic alteration was found in 10 cases. The most common co-occurring mutations were *EGFR* and *TP53* in 70% (7/9), followed by *EGFR* and *KRAS G13D* in 20% (2/10) and 1 case with *ALK* rearrangement and *TP53* was observed. The median PFS was 9.8 months (95% CI 7.5–12.1) in *EGFR*-positive patients (without concomitant mutations), compared with 8.7 months (95% CI 0–19.9; $p = 0.049$) in patients with an *EGFR-TP53* concurrent mutation. The median OS was 48.5 months (95% CI 19.5–77.4) in *EGFR*-positive patients (without concomitant mutations) and 12.6 months (95% CI 5.7–19.6; $p < 0.001$), in patients with an *EGFR-TP53* concurrent mutation (Fig. 2).

Number of Genetic Alterations and Outcomes

Among the 52 patients with a cfDNA test, 46% ($n = 24$) had only 0–2 alterations per test, while 54% ($n = 28$) presented with 3 or more alterations per test. No differences were observed in the median PFS. The median OS was 46.7 (95% CI 35.1–58.3) and 18.6 months (95% CI 8.9–28.4) in patients with 0–2 and 3 or more alterations per test, respectively ($p = 0.002$; Fig. 3).

Discussion

Liquid biopsy from peripheral blood offers a unique opportunity not only for the longitudinal tumor monitoring in a non-invasive fashion, but also as a diagnostic alternative for patients whose tissue biopsy had insufficient material for through testing, or when their clinical condition does not allow for tissue acquisition. In this

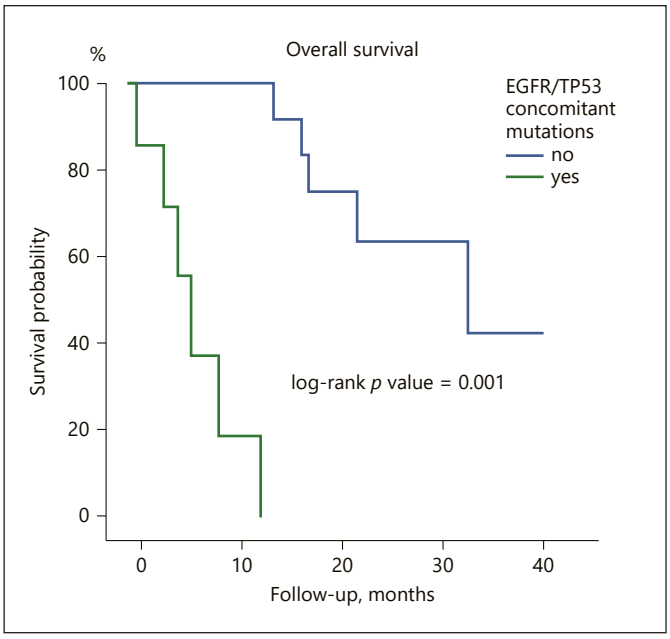
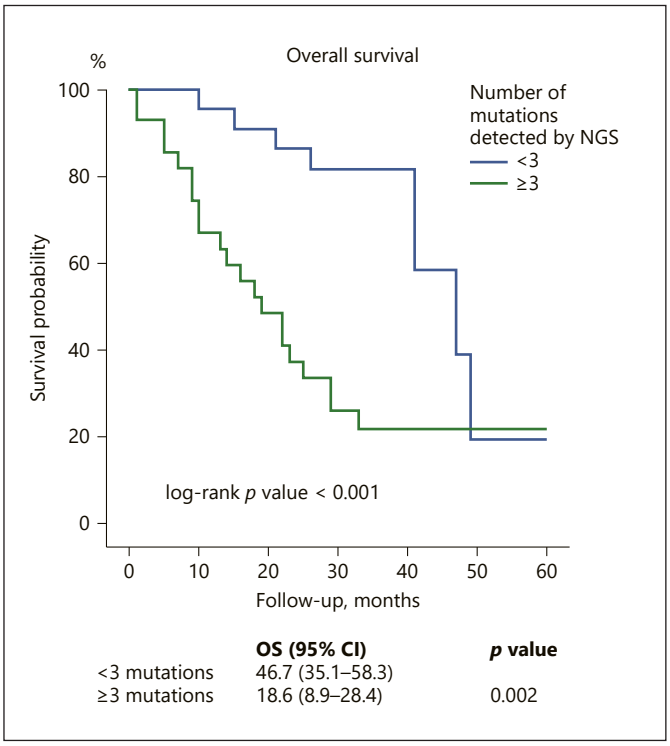


Fig. 2. OS in patients with a mutated *EGFR* and wild-type *TP53* profile compared with patients with concomitant *EGFR*/*TP53* mutations.



	OS (95% CI)	*p* value
<3 mutations	46.7 (35.1–58.3)	
≥3 mutations	18.6 (8.9–28.4)	0.002

Fig. 3. OS in patients with less than three alterations compared with those with ≥3 mutations.

study, the results showed that many advanced lung adenocarcinoma patients who were previously negative for important biomarkers such as *EGFR* and *ALK* via tissue-based testing, were able to receive targeted therapy due to positive cfDNA results. The International Association for the Study of Lung Cancer (IASLC) recommends the use of cfDNA analysis as an alternative to tumor tissue biopsy and its results as sufficient information in order to initiate targeted therapy [16]. In routine diagnostics, the cancer specimens are FFPE samples, which yield DNA in a limited quantity due to degradation or an unobtainable status (reported failure rates range from <10 to >30% of cases). In our study the genomic profile in tissue biopsy in FFPE failed to accurately diagnose several targetable alterations (either due to false negatives or technical limitations such as insufficient or low-quality DNA). Contrary, cfDNA was able to detect different genomic alterations previously undetected by tissue biopsy.

Conventional methods for liquid biopsy analysis, including real-time or quantitative PCR have limited precision, especially due to their low sensitivity and high proportion of false negatives, and often do not test for all of the potentially targetable mutations in these genes. Novel technologies, such as NGS, have overcome this limitation. Additional advantages of NGS approaches with specific regard to cfDNA include the ability to quantitate gene copy number variations, including gene amplification, and to identify chromosomal rearrangements such as oncogenic fusions. Guardant360 is an NGS panel which sequences 74 genes utilizing cell-free ctDNA isolated from a simple, less-invasive blood draw [10]. In previous studies, Raez et al. [17] reported that 47 (28%) of 171 patients had at least one actionable mutation identified on Guardant360 at multiple clinical time points. Furthermore, Leighl et al. [18] recently reported that among a population of patients with newly diagnosed metastatic NSCLC, cfDNA increased the detection of identified targetable biomarkers (*EGFR*, *ALK*, *ROS1*, *BRAF*, *RET*, *MET*, *ERBB2*) and rescued 30.2% (85/282) of patients, including those who had an insufficient amount of tissue for testing, and those who were incompletely genotyped or who were negative for the biomarker in tissue. In this study, we tested, among others, *EGFR* and *ALK* status through cfDNA and identified alterations which led to a therapy switch in 28.8% (n = 15) and 3.8% (n = 2) of patients. Patients whose samples have these characteristics are usually cataloged as having an undetermined status for driver mutations, which unfortunately often renders them ineligible for receiving targeted therapy. Moreover,

we identified other genomic alterations that are not routinely tested in tissue biopsy and which also led to a therapy switch, including *BRAF*, *ERBB2* (HER2), and *ROS1*. The results from this high-sensitivity analysis resulted in a changed treatment strategy for 46% of our patients with cfDNA alterations (n = 24). Furthermore, these patients had a high response rate to their new targeted therapy and presented a favorable OS after a switch in therapy based on cfDNA test results. Despite the encouraging data, further studies must be conducted in order to be able to make further comparisons regarding survival among patients who receive targeted treatment due to the identification of specific molecular targets through NGS testing.

Several practice guidelines recommend comprehensive genomic profiling in lung cancer beyond just the common *EGFR* mutations or *ALK* rearrangements, particularly when taking into account that new therapies are available for other specific genomic alterations [19, 20]. As an example, *BRAF* evaluation is recommended; approximately 0.5–4.9% of lung adenocarcinomas have *BRAF* alterations and two phase II trials showed a significant response with targeted therapies in this setting that led to a breakthrough therapy designation conferred by the FDA for the combination treatment of *BRAF* p.V600E mutation-positive NSCLC, leading to approval in 2017 [20–22]. In our study we identified *BRAF* mutations that could be potentially treated with targeted agents. Interestingly, the incidence of *ALK* rearrangements in Latin America is estimated at 6.8%, nonetheless this can vary according to each specific country, and in the case of Mexico this figure is of 7.6% in the general population while in Colombia the reported incidence is 4.1% in the general population. The incidence of ALK fusions in this specific study should, nonetheless, be interpreted considering the highly selected population [23].

KRAS-mutant lung cancer is common, but treatment options are still limited, and chemotherapy remains the first-line recommendation [24]. However, many clinical trials are ongoing. A phase I clinical trial (NCT03600883) involving a KRASG12C inhibitor, AMG 510, in 32 patients with a *KRASG12C* mutation (14 with NSCLC), showed that 5 out of 10 evaluable patients with NSCLC had a PR and 4 had SD [25]. Trametenib has also been tested in KRAS-mutant NSCLC. A phase II study with 54 *KRAS*-mutant NSCLC patients showed a trend toward worse PFS and OS in G12C versus non-G12C patients [26]. Furthermore, the presence of a *KRAS* mutation generally predicts the absence of other targetable NSCLC biomarkers due to mutual exclusivity. Therefore, although it is not currently an actionable finding, identification of a *KRAS* mutation essentially rules out the presence of other actionable driver mutations in treatment-naïve patients.

Concomitant mutations could potentially affect the activity of EGFR TKIs. In a large retrospective study in 3,774 NSCLC patients, 1.7% harbored mutations in two or three genes. Among these patients, *EGFR*/*KRAS* was the most frequent co-alteration in 31.7% of cases, followed by *ALK*/*KRAS* in 17.5% of cases. Interestingly, in this study *KRAS* alterations had no effect on EGFR-TKI therapy [27]. Another study with 133 patients tested with NGS reported a frequency of concomitant mutations of 21.8% and their presence was associated with a worse PFS. Investigators report that no correlation was found with *TP53* mutations [28]. In this study, we identified 10 patients harboring concomitant alterations. The most common concurrent mutations in our study were *EGFR-TP53* in 13.5% (n = 7/52), followed by *EGFR-KRAS* G13D in 3.8% (n = 2/52). Concomitant *TP53* mutations have been previously described. In a study with 71 advanced NSCLC patients, a *TP53* mutation was present in 60.6% and was associated with a worse median PFS (6.5 vs. 14 months; p = 0.025) and median OS (28 vs. 52 months; p = 0.023). *TP53* with non-missense mutations and those affecting exon 6 and 7 lead to worse PFS and OS outcomes [29]. Another retrospective study in 33 patients with advanced lung adenocarcinoma harboring sensitizing *EGFR* mutations found concomitant genetic alterations in 78.8% of cases, with the *EGFR-TP53* presenting as the most common concurrent mutation, followed by *CDK4* and *CDK4A*. Patients with concomitant genetic alterations had a worse OS (24.1 vs. 40.8 months; p = 0.029) [30]. These retrospective studies have similar outcomes in patients with concomitant genetic mutations. Nonetheless, both these studies were performed in Asian populations, and therefore the effect of this finding in Hispanics has never before been reported, to the best of our knowledge.

In limited resource settings, such as in Latin American countries, a frequent scenario that clinicians will face is an NSCLC patient without an adequate or sufficient tumor tissue for a complete histological and genomic analysis. This is a particular problem for lung adenocarcinoma, in which international guidelines recommend assessment of genomic alterations in seven different targetable genes (*EGFR*, *ALK*, *HER2*, *BRAF*, *MET*, *ROS1*, and *RET*) to guide different targeted therapies [17, 18]. In our study, liquid biopsy identified *EGFR* mutations or *ALK* rearrangements that were not found in tissue analyses, allowing clinicians to arrive to an accurate diagnosis and make

treatment modifications according to these results. It is important to highlight that the patients included in this cohort reside in areas where access to genomic profiling and to targeted therapy is often very limited. Patients regularly receive chemotherapy without previous molecular characterization, and even when tested they frequently lack access to newer targeted agents. This represents an important limitation, as patients have limited access to tissue molecular characterization.

Although the timing of cfDNA determination was associated with OS, it is important to highlight that the population was comprised of patients with diverse mutation profiles and previous lines of therapy. This information likely results from a selection bias with respect to the type of patients who are referred to the thoracic oncology unit at the National Cancer Institute of Mexico. Such patients are frequently heavily pretreated patients who are seeking to enter a clinical trial; therefore, the data must be interpreted with caution.

The present study emphasizes the importance of early detection of diverse molecular profiles (i.e., genomic alterations, concomitant mutation) which can be therapeutically relevant, which should allow a better selection of patients who are candidates for approved and available therapies. Liquid biopsy plays an important role in the genomic analysis of metastatic NSCLC, offering reliable information to guide therapeutic decision making.

Conclusions

In a selected population of Hispanic NSCLC patients, the use of comprehensive cfDNA analysis allowed a treatment change in 46.1% of the cases. Our data support the usefulness of Guardant360 as a non-invasive panel to identify genomic alterations that could lead to treatment change to improve oncologic outcomes. Further prospective studies are needed to confirm the treatment outcomes of patients harboring a *TP53* or *KRAS* combined with an *EGFR* mutation.

Acknowledgements

We acknowledge the support from all the patients who participated in this study.

Statement of Ethics

This study was performed in accordance with the World Medical Association Declaration of Helsinki, and was approved by the local research and ethics committee at the participating institution (015/049/ICI; CEI/1023/15). The patients provided written informed consent for inclusion in the study and for provision of tumor and plasma samples.

Conflict of Interest Statement

Rebecca Nagy and Jennifer Saam report being employees at Guardant Health.

Funding Sources

The material to perform the genotyping of cell-free circulating tumor DNA was donated by Guardant Health.

Author Contributions

A.F.C., G.C.-R., F.B., and O.A. conceived and designed the analysis. Z.L.Z.-B., D.D.-G., R.T.R., L.R., G.C.-R., L.C., and C.V. collected the data. A.F.C., G.C.-R., R.N., J.S., F.B., and O.A. contributed data or analysis tools. A.F.C., Z.L.Z.-B., F.B., O.A., and G.C.-R. performed the analysis. All authors contributed to manuscript writing and editing.

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