ORIGINAL RESEARCH ARTICLE

Genomic Landscape of Primary Resistance to Osimertinib Among Hispanic Patients with *EGFR*-Mutant Non-Small Cell Lung Cancer (NSCLC): Results of an Observational Longitudinal Cohort Study

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Abstract

Background Epidermal growth factor receptor (*EGFR*) mutations (*EGFR*m) represent one of the most common genomic alterations identified among patients with non-small cell lung cancer (NSCLC). Several targeted agents for patients with *EGFR*m have been proven safe and effective, including the third-generation tyrosine kinase inhibitor (TKI) osimertinib. Nonetheless, some patients will present with or develop EGFR-TKI resistance mechanisms.

Objective We characterized the genomic landscape of primary resistance to osimertinib among Hispanic patients with *EGFR*-mutant NSCLC.

Methods An observational longitudinal cohort study was conducted with two groups of patients, those with intrinsic resistance (cohort A) and those with long-term survival (cohort B). All patients were treated and followed between January 2018 and May 2022. All patients were assessed for Programmed Cell Death Ligand 1 (PD-L1) expression and Bcl-2-like protein 11 (BIM)/*AXL* mRNA expression before starting TKI. After 8 weeks of treatment, a liquid biopsy was performed to determine the presence of circulating free DNA (cfDNA), and next-generation sequencing (NGS) was used to identify mutations at the time of progression. In both cohorts, overall response rate (ORR), progression-free survival (PFS), and overall survival (OS) were evaluated.

Results We found a homogeneous distribution of *EGFR*-sensitizing mutations in both cohorts. For cohort A, exon 21 mutations were more common than exon 19 deletions (ex19dels) for cohort B ($P = 0.0001$). The reported ORR for osimertinib was 6.3% and 100% for cohorts A and B, respectively ($P = 0.0001$). PFS was significantly higher in cohort B (27.4 months vs. 3.1 months; $P = 0.0001$) and ex19del patients versus L858R (24.5 months, 95% confidence interval [CI] 18.2–NR), vs. 7.6 months, 95% CI 4.8–21.1; $P = 0.001$). OS was considerably lower for cohort A (20.1 months vs. 36.0 months; $P = 0.0001$) and was better for patients with ex19del, no brain metastasis, and low tumor mutation burden. At the time of progression, more mutations were found in cohort A, identifying off-target alterations more frequently, including *TP53*, *RAS,* and *RB1*.

Conclusion *EGFR*-independent alterations are common among patients with primary resistance to osimertinib and significantly impact PFS and OS. Our results suggest that among Hispanic patients, other variables associated with intrinsic

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resistance include the number of commutations, high levels *AXL* mRNA, and low levels of *BIM* mRNA, T790M de novo, *EGFR* p.L858R presence, and a high tumoral mutational burden.

Key Points

The prognosis of patients with *EGFR* mutations and primary resistance to osimertinib is utterly opposed to those with a high sensitivity to osimertinib.

Patients with *EGFR* mutations with complex genotypes, including alterations in *TP53* and *RB1*, and a high tumor mutation burden, require a different therapeutic approach to osimertinib as the first-line intervention.

Early assessment of *EGFR* circulating free DNA (cfDNA) allows prediction of the disease course in patients with primary resistance to osimertinib. This information could allow the use of risk-adaptive therapeutic models.

AXL mRNA expression levels are considerably higher among patients with *EGFR* mutations and primary resistance to osimertinib. In contrast, Bcl-2 interacting mediator of cell death (*BIM*) mRNA expression levels were significantly lower in cohort A.

1 Introduction

Alterations in the epidermal growth factor receptor (*EGFR*) gene are among the most common oncogenic driver mutations in the pathogenesis of non-small cell lung cancer (NSCLC). Specific mutations in the *EGFR* gene are associated with increased sensitivity to EGFR tyrosine kinase inhibitors (EGFR-TKI), which offer the best possible results for treatment response in patients with an NSCLC diagnosis and *EGFR* mutations (*EGFR*m) [1, 2]. *EGFR*m presence has been reported in 10–50% of NSCLC cases, almost all in lung adenocarcinomas (LUADs). In addition, significant ethnic variations for *EGFR*m prevalence have been described, such as 8–15% in LUADs diagnosed in Caucasian patients versus 30–50% in East Asian populations [3, 4]. Among Hispanic populations in Latin America, the frequency of *EGFR*m in LUADs ranges from 14% in Argentina to 24–35% in Colombia and as high as 50% among Peruvian populations [5–7]. Evaluation of ancestry–mutation association showed that Native American ancestry is positively correlated with *EGFR*m [8, 9].

Among the EGFR-TKI-sensitizing mutations, exon 19 deletions (ex19dels) in the p.E746-A750 region and the point-substitution p.L858R (L858R) in exon 21 are the two most commonly described [10, 11]. These alterations represent ≈90% of EGFR-TKI-sensitizing mutations [12]. ex19dels and L858R are early clonal events that lead to tumor development in LUADs. They result in constitutive ligand-independent EGFR-tyrosine kinase activity and an increased affinity for first-generation (i.e., gefitinib, erlotinib, and icotinib), second-generation (i.e., afatinib and dacomitinib), and third-generation (i.e., osimertinib and aumolertinib) EGFR-TKI [13]. Nowadays, the National Comprehensive Cancer Network recommends osimertinib as the preferred EGFR-TKI option as first-line treatment for NSCLC because it inhibits both EGFR-TKI-sensitizing and the *EGFR* resistance mutation p.Thr790Met (T790M), and it has shown efficacy in patients with central nervous system (CNS) metastases [14].

The FLAURA trial (NCT02296125) recruited over 550 patients with ex19dels or L858R *EGFR*-mutated advanced NSCLC, from December 2014 to March 2016 [15]. In this study, the individuals were stratified according to *EGFR*m status and ethnicity (Asian/non-Asian), then half of the patients were treated with oral osimertinib and the other half were treated with other TKI [16–18]. After follow-up, the study demonstrated a better overall survival (OS) and a favorable OS rate in the group treated with osimertinib. Although the FLAURA trial and other studies have consistently shown the superior efficacy of osimertinib in comparison with first- and second-generation EGFR-TKI, some patients develop resistance mechanisms to third-generation TKI. These resistance mechanisms affect a patient's prognosis under TKI treatment. Most patients with advanced *EGFR*m NSCLC will achieve an objective response after treatment with TKI, but will eventually present disease progression. The extent and duration of responses are variable. However, approximately 10–15% of patients do not respond to treatment or have control of the disease for a short time, usually less than 3 months [19, 20].

Current evidence suggests two types of resistance mechanisms: *EGFR* dependent and *EGFR* independent. Some *EGFR*-dependent resistance mechanisms have been described for first-line and second-line treatment, including acquired *EGFR*m (C797S, L718/G719, G796/C797, L792, L798, and *EGFR* amplification). These profiles pose a significant challenge in selecting the most effective treatment based on resistance patterns after disease progression. Regarding the *EGFR*-independent mechanisms of acquired resistance, the activation of bypass pathways via amplifications (i.e., epithelial growth factor receptor or ERBB2/HER2, MET, fibroblast growth factor receptor 1 or FGFR1, and KRAS), fusions of alternative receptor tyrosine kinase

(RTK) genes (i.e., RET, anaplastic lymphoma kinase [ALK], fibroblast growth factor receptor 3 [FGFR3], and neurotrophic receptor tyrosine kinase 1 [NTRK1]), as well as activating mutations and fusions of members of the downstream RAS-RAF-MEK-MAPK and PI3K/AKT/PTEN/mTOR pathways have been proposed as possible explanations [21]. Additional mechanisms of acquired resistance shared by TKI of all three generations include the phenotypic transformation to small-cell lung cancer (SCLC), epithelial-mesenchymal transition (EMT), the conversion to squamous cell carcinoma (SCC), activation of the Wnt/β-catenin pathway, hormonal signaling, and cell cycle were observed in some *EGFR* mutant cases compared to wild-type EGFR cases [22–25].

It has been proposed that acquired resistance could emerge from the expansion of original pre-treatment resistant clones and new resistance mechanisms developed as an adaptative response to TKI exposure. On the other hand, intrinsic resistance mechanisms are still a matter of debate and secondary activating pathways remain poorly understood [19, 20, 26]. Herein, we characterize the clinical, molecular, and genomic features associated with an intrinsic resistance pattern to osimertinib therapy in treatment-naïve patients with *EGFR*m NSCLC [24, 25].

2 Methods

2.1 Study Design and Patients

An observational longitudinal cohort study was conducted by the Latin American Consortium for Investigation of Lung Cancer (CLICaP) using the data deposited in a centralized database (CLICaP Real World Data/Evidence database). Patients included in the *EGFR*m NSCLC database were assessed from January 2018 to May 2022. All patients were residents of Bogota, DC, Colombia, and demographic variables such as age, sex, and smoking history were collected from the clinical record. Eligible patients were those with *EGFR*m analyzed by molecular diagnosis ($N = 578$) (Fig. 1). Among these patients, we defined cohort A as cases with intrinsic resistance to first-line osimertinib with measurable disease progression within the first 3 months of treatment ($N = 16$). In contrast, cohort B were patients with long-term responses to osimertinib treatment or progression-free survival (PFS) with osimertinib > 18 months. Finally, this control group was selected from a cohort of long survivors with genomic evaluation by next-generation sequencing (NGS) and complete clinical follow-up. Cases with similar clinical characteristics were sought without being able to match them, due to the unusual nature of patients with primary resistance.

At diagnosis, tumor tissue was acquired from all included patients to undergo molecular characterization, including PD-L1 expression by immunohistochemistry (IHC) and a standardized NGS test. A liquid biopsy was also obtained before starting osimertinib to evaluate Bcl-2 interacting mediator of cell death (*BIM*) polymorphisms. In the baseline tumor tissue, *BIM* and *AXL* mRNA expression levels were quantified using reverse transcription-polymerase chain reaction (RT-PCR). Cohort A and B patients received first-line osimertinib (80 mg orally once daily) until disease progression or unacceptable toxicity was documented. Patients were followed for 48 months and assessed for progression. After 8 weeks of treatment with osimertinib, a new liquid biopsy was performed to estimate the *EGFR*m status by droplet digital PCR (ddPCR). The NGS test in liquid biopsy was repeated when progression was found, and tissue collection was attempted when there was suspicion of histological transformation (SCLC). The study design and patient inclusion are summarized in Fig. 1.

2.2 Follow-Up and Clinical Outcomes

Patients included in the study were assessed clinically every month. Radiological follow-ups were completed every 9–12 weeks. Clinical, demographic, and molecular variables (including all mutations and tumor mutational burden [TMB]) were stored in a centralized and deidentified database at the CLICaP/Foundation for Clinical and Applied Cancer Research—FICMAC (Bogotá, Colombia). All included patients provided signed informed consent. In addition, an Institutional Review Board and Privacy Board waiver was obtained to facilitate retrospective clinical-pathological and molecular data (Lung Cancer-FICMAC/CLICaP Platform—Registration No. 2012/014, Kayre, Bogotá, Colombia, and Research Ethics Committee of Universidad El Bosque—Registration No. PCI-2018-10171).

Overall response rate (ORR) was defined as the sum of complete responses (CRs) and partial responses (PRs), as assessed by an independent, blinded radiologist according to the Response Evaluation Criteria in Solid Tumors (RECIST) V1.1. For PFS, time to event was calculated from the date of treatment start until radiographic disease progression as assessed by a blinded radiologist according to RECIST V1.1 or death by any cause. Observations for patients who did not experience progression were censored at the patient-specific last follow-up. OS was estimated from diagnosis until death by any cause or loss to follow-up.
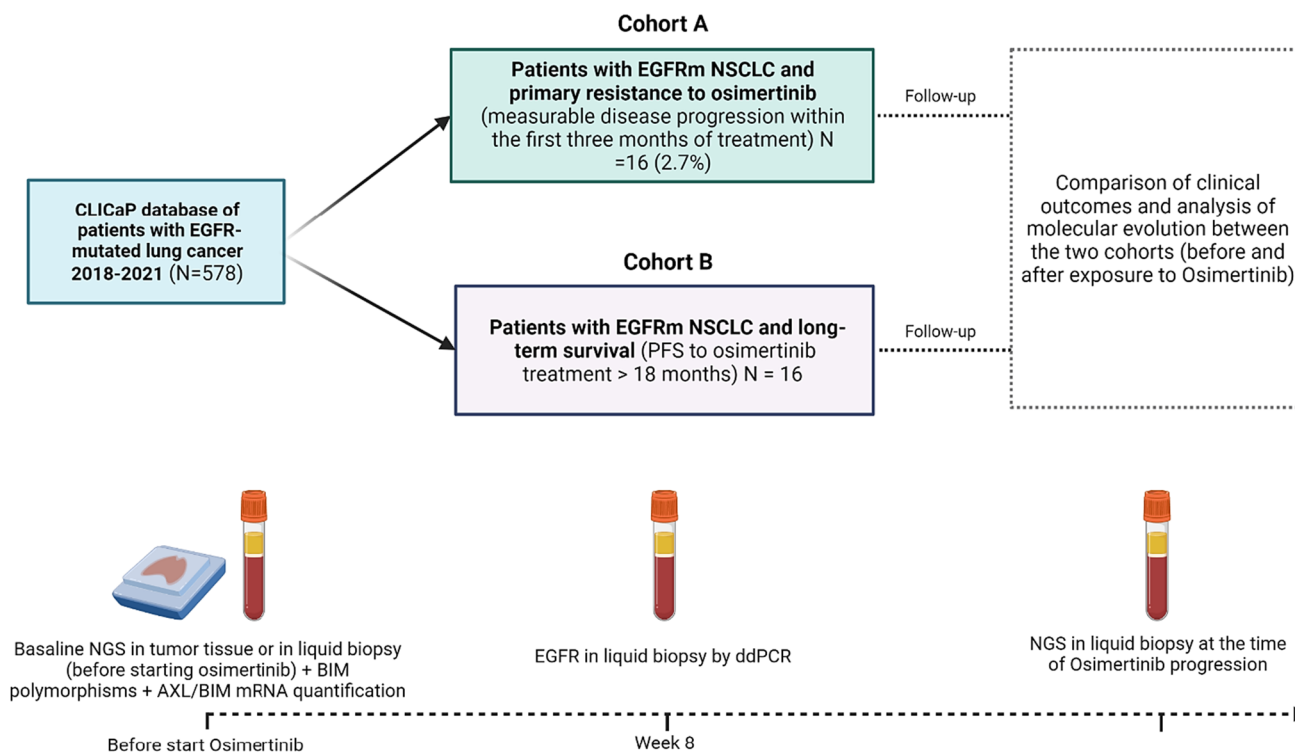
Fig. 1 The design and distribution of the study cohorts. We identified 578 potentially eligible patients. Cases with intrinsic resistance to first-line osimertinib (measurable disease progression within the first 3 months of treatment) were identified (cohort A). Control group patients were selected for convenience from a cohort of long survivors with genomic evaluation by NGS and complete clinical follow-up. Cases with similar clinical characteristics were sought without being able to match them due to the unusual nature of patients with primary resistance (cohort B). *BIM* Bcl-2 interacting mediator of cell death, *ddPCR* droplet digital polymerase chain reaction, *EGFR* epidermal growth factor receptor, *EGFRm* epidermal growth factor receptor mutation, *NGS* next-generation sequencing, *NSCLC* non-small cell lung cancer, *PFS* progression-free survival

2.3 PD-L1 Testing

PD-L1 expression was determined by IHC using the Dako 22C3 pharmDx kit, considering more than 100 tumor cells in the slide section for accurate PD-L1 readings. PD-L1 testing was completed on biopsies taken at diagnosis. For survival analyses, patients were stratified according to PD-L1 status (i.e., $\geq 50\%$, 1–49%, and $< 1\%$ subgroups). Patients with unknown PD-L1-expression status were also included in this study.

2.4 cfDNA Detection (Week 8) by Droplet Digital PCR Analysis

The first blood sample was collected at the time of diagnosis before the start of treatment. Circulating free DNA (cfDNA) was quantified using the QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA). For the EGFR multiplex assays, a final PCR mix volume of 20 µL was manually loaded into wells of a DG8 cartridge (Bio-Rad Laboratories) with 70 µL of Droplet Generation Oil for

probes (Bio-Rad Laboratories). After droplet generation by the QX200 Droplet Generator (Bio-Rad Laboratories), 40 µL of the sample was transferred into a 96-well PCR plate and amplified with a C1000 Touch Thermal Cycler (Bio-Rad Laboratories), using the following thermal cycling conditions: 95 °C for 10 min, 40 cycles at 94 °C for 30 s, 55 °C for 1 min (2 °C/s), and 98 °C for 10 min, and a final cooling step to 12 °C (1 °C/s). The droplets were analyzed using the QX200 Droplet Reader (Bio-Rad Laboratories), and data were analyzed using QuantaSoft software version 1.7.4.0917 and QuantaSoft Analysis Pro software version 1.0.596 (Bio-Rad Laboratories). Thresholds were placed manually, and the fractions of positive and negative droplets were used to calculate the concentration and fractional abundance of target DNA sequences with their 95% Poisson-based confidence intervals (CI). *EGFR*m primers and probes were based on previous studies [27, 28].

2.5 AXL and BIM mRNA Quantification by RT-PCR Analyses

Samples were lysed in a buffer containing EDTA, SDS, and proteinase K. Then RNA was extracted with phenol-chloroform-isoamyl alcohol, followed by precipitation with isopropanol in the presence of glycogen and sodium acetate. RNA was homogenized in water and treated with DNAse I to avoid DNA contamination. Complementary DNA (cDNA) was synthesized using M-MLV (Moloney Murine Leukemia Virus Reverse Transcriptase) retro-transcriptase enzyme. cDNA was added to Taqman Universal Master Mix in a 12.5-μL reaction with specific primers and probes for each gene. The primer and probe sets were designed using the LightCycler® Probe Design Software 2.0 (Roche Diagnostics Corporation, Indianapolis, Indiana) according to their Ref Seq (National Center for Biotechnology Information). Quantification of gene expression was performed using the LightCycler® 480 Real-Time PCR System (Roche Diagnostics Corporation, Indianapolis, Indiana) and calculated according to the comparative Ct method. The final results were determined as follows: 2-(ΔCt sample-ΔCt calibrator), where ΔCt values of the calibrator and sample are determined by subtracting the Ct value of the target gene from the value of the endogenous gene (β-actin). Commercial RNA controls were used as calibrators (Stratagene, La Jolla, CA, USA). In quantitative experiments, a sample was considered non-viable when the standard deviation (SD) of the Ct values was ≥ 0.30 in two independent analyses. To dichotomize the mRNA expression levels, *AXL* 1.42 (high *AXL* [*AXL*-H] ≥ 1.42) and *BIM* 0.98 (high *BIM* [*BIM*-H] ≥ 0.98) were used as cut-off points; the cut-off points are based on the values described in the existing evidence [29, 30].

2.6 Next-Generation Sequencing

cDNA synthesis prior to library preparation for the RNA panel was carried out using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, 2018, 11754050). Library preparation was carried out using the Oncomine™ Comprehensive Assay Plus (Thermo Fisher Scientific, A49667) and RNA Oncomine™ Fusion's assay (Thermo Fisher Scientific) kits following the instructions of the manufacturer and using a total of 10 ng input DNA and/or RNA per sample (minimum 0.83 ng/μL sample DNA concentration). A maximum of seven DNA samples were prepared per run (six samples if both DNA and RNA analyses were required) on an Ion 540™ Chip (Thermo Fisher Scientific, A27765) using the Ion S5™ sequencing system (see the electronic supplementary material, Supplementary Data 1 and 2). The DNA panel can identify hotspot mutations and copy number variations (CNVs), full coding DNA sequences (CDSs), and RNA fusions (Supplementary Table 1 shows the genes and alterations included in the analysis). Template preparation was performed on the Ion Chef System (Thermo Fisher Scientific) using the Ion PGM Hi-Q Chef Kit and/or the Ion One Touch™ 2 System using the Ion PGM Template OT2 200 Kit. Sequencing was performed using the Ion PGM Hi-Q Sequencing Kit on the Ion Torrent Personal Genome Machine. TMB was measured by running the Oncomine Tumor Mutation Load Assay (Thermo Fisher Scientific, A37909), which enables accurate measurements of somatic mutations without needing a matched normal sample. This assay covers a large genomic footprint of 1.7 Mb, encompassing 1.2 Mb of exonic sequence, to enable accurate mutation counts for samples with a range of TMBs. For the analysis, TMB–high (TMB-H) was defined as ≥ 10 mutations/megabase [mut/Mb]) using the same cut-off as in the KEYNOTE-158 study (NCT02628067) [31].

2.7 Next-Generation Sequencing Data Analysis

Ion Torrent Suite™ Browser version 5.0 and Ion Reporter™ version 5.0 were used to analyze the NGS data. The Torrent Suite™ Browser was used to perform initial quality control, including chip loading density, median read length, and the number of mapped reads. The Coverage Analysis plugin was applied to all data to assess amplicon coverage for regions of interest. Variants were identified by the Ion Reporter filter chain 5% Oncomine™ Variants (5.0). A cut-off of 500X coverage was applied to all analyses. All identified variants were checked for correct nomenclature using Alamut Visual v.2.7.1 (Interactive Biosoftware). Any discrepancies in variant identification between Ion Reporter and Alamut were validated manually using the Integrative Genomics Viewer and NextGENe® v2.4.2 (SoftGenetics®) [32, 33].

2.8 Statistical Analysis

All analyses were conducted on IBM SPSS Statistics software version 25.0 (SPSS Inc. Chicago, IL, USA). Descriptive analyses were used to provide an overview of the study population characteristics. Categorical variables were assessed via the Chi-square test or, whenever appropriate, Fisher's Exact test. OS and PFS were reported as Kaplan–Meier survival curves. Univariable regression analysis models were generated to assess potential confounders. Mann–Whitney *U* test with a two-sided *P* value was established to determine statistically significant outcomes. There were no adjustments made for multiple comparisons, and in all cases, the significance level was 0.05.

3 Results

3.1 Baseline Patient Characteristics

The clinical information derived from 32 Hispanic patients with NSCLC and *EGFR*m is summarized in Table 1. Demographic characteristics indicate that all patients were diagnosed at stage IV, and most patients had an adenocarcinoma histology type (96.9%), mainly solid or lepidic patterns (56.3%). There is a higher proportion of female patients in both cohorts (60%), and at least 65% had a negative smoking history. Most patients had pleuropulmonary compromise (87.5%) and good functional status (Eastern Cooperative Oncology Group, ECOG performance status 0–1, 90.7%). Samples were obtained from the lung or pleural space in 90.6%. Most patients had a liquid biopsy at progression (96.9%), and in two cases, tumor tissue was obtained by repeated biopsy. Both cases confirm the suspicion of differentiation to SCLC. Overall, the distribution of *EGFR*-sensitizing mutations was comparable in the population, including ex19dels and L858R mutations. However, patients in cohort A had significantly more frequent alterations in exon 21 than in cohort B in exon 19 ($P = 0.0001$). Seven patients had secondary *EGFR*m (21.9%), which occurred more frequently in cohort B ($P = 0.32$). A baseline T790M mutation was identified in five cases, four in cohort A ($P = 0.16$), with a mean allele frequency of 39 (SD $\pm$ 20). Thirteen patients (87.5%) in cohort A had more than three co-mutations at the time of diagnosis, a significantly higher finding than for cohort B ($P = 0.0001$). Almost all baseline *TP53* commutations (82.4%) and those involving the RAF pathway were identified among patients in cohort A ($P = 0.001$ and $P = 0.009$). Besides *EGFR* and *TP53* genes, the main commutations identified at baseline included *RB1, AT-rich interactive domain-containing protein 1A [ARID1A],* and *BRAF.* Fig. 2 shows the primary baseline genomic alterations identified in both cohorts, and Supplementary Table 2 details the variants detected at diagnosis (see the electronic supplementary material).

The baseline TMB was obtained in 27 patients, among whom 22 (68.8%) had a low TMB (TMB-L). It is important to note that cohort A included five patients (31.3%) with TMB-H, while in cohort A, all patients with available data (11/16; 68.8%) had TMB-L ($P = 0.054$). Based on the mRNA levels stratification, there was a statistically significant difference when *AXL* and *BIM* levels were compared among both cohorts (81.3%, $N = 13$, cohort A patients had *AXL*-H; 93.8%, $N = 15$, had *BIM*-H; 75%, $N = 12$, cohort B patients had low *AXL* [*AXL*-L]; 85.7%, $N = 14$, had low *BIM* [*BIM*-L]). As shown in Supplementary Fig. 1, both differences in mRNA levels between cohorts A and B were statistically significant. Monitoring of response to osimertinib

using ddPCR multiplex assays performed at week 8 was positive in 18 patients (56.3%); 93.8% and 18.8% of patients in cohorts A and B had cfDNA detectable by ddPCR, and this difference was statistically significant ($P = 0.0001$; Supplementary Fig. 2). In terms of PD-L1, most patients had < 1% ($N = 3$; 18.8%) and 1–49% ($N = 11$; 68.8%) in cohort A. Similarly, in cohort B, most patients had < 1% ($N = 6$; 37.5%) and 1–49% ($N = 10$; 62.5%). Only two patients had PD-L1 $\geq$ 50% in the study population, both from cohort A.

3.2 Outcomes Obtained with First-Line Osimertinib

The median time between the first sample and the start of therapy was 15 days, and the median time between the start of therapy and the first follow-up evaluation was 60 days ($\pm$ 4 days). The ORRs for cohorts A and B were 6.3% and 100.0% ($P = 0.0001$), respectively. Only one of the patients with primary resistance to osimertinib had a short-lived PR, with subsequent multifocal progression, including the brain. On the contrary, in cohort B, 31.3% achieved a CR and 68.8% a PR. In both cohorts, the leading site of progression was the lung ($\approx$60%), and cohort A patients had twice as many brain metastases, generally in the first 6 months after diagnosis ($P = 0.67$). PFS with osimertinib was 3.1 months (95% CI 0.47–7.0) for cohort A and NR for cohort B ($P = 0.0001$) (Fig. 3A). PFS was significantly higher in patients with ex19del compared with those with L858R mutations (24.5 months, 95% CI 18.2–NR, vs. 7.6 months, 95% CI 4.8–21.1; $P = 0.001$) (Supplementary Fig. 3; see the electronic supplementary material) and in those without brain metastases at diagnosis compared with patients with baseline brain metastases (24.5 months, 95% CI 18.5–30.5, vs. 7.2 months, 95% CI 1.1–13.2; $P = 0.004$) (Supplementary Fig. 4). The baseline T790M mutation also negatively impacted PFS (Supplementary Fig. 5); similarly, the presence of mutations in *TP53* ($P = 0.001$), a higher number of commutations at diagnosis ($P = 0.0001$; Supplementary Fig. 6), and TMB-H ($P = 0.017$; Supplementary Fig. 7) were associated with worse PFS. In contrast, the PD-L1 expression level did not significantly affect PFS ($P = 0.36$). Given the biological differences between cohort A and B, high mRNA levels of *AXL* ($P = 0.0001$) and low *BIM* ($P = 0.001$) also affected PFS, as did the presence of cfDNA in liquid biopsies obtained 8 weeks after starting treatment with osimertinib ($P = 0.001$).

After a median follow-up of 25.1 months (95% CI 11.0–43.0), the OS for cohorts A and B was 20.1 months (95% CI 10.4–29.2) and NR ($P = 0.0001$, Fig. 3B). OS was higher for patients with ex19del ($P = 0.002$; Supplementary Fig. 3B), those without baseline brain metastases ($P = 0.013$; Supplementary Fig. 4B), those with two or fewer commutations ($P = 0001$; Supplementary Fig. 6B), and those with TMB-L ($P = 0.0001$; Supplementary Fig. 7B).

Table 1 Baseline characteristics of patients included in the study

Variable	Overall population (N = 32)	Cohort A (N = 16)	Cohort B (N = 16)
Age at diagnosis			
Mean ± SD	58 ± 10.7	61 ± 12.1	55 ± 8.4
Median (range)	60 (32–79)	64 (32–79)	51 (44–69)
Sex			
Male	13 (40.6)	8 (50.0)	5 (31.3)
Female	19 (59.4)	8 (50.0)	11 (68.8)
Smoking history			
Never smoker	20 (62.5)	8 (50.0)	12 (75.0)
Former smoker	11 (34.4)	7 (43.8)	4 (25.0)
Current smoker	1 (3.1)	1 (6.3)	–
Histology at diagnosis			
Adenocarcinoma	31 (96.9)	15 (93.8)	16 (100.0)
NOS	1 (3.1)	1 (6.3)	–
Histological subtype			
Papillary	5 (15.6)	1 (6.3)	4 (25.0)
Micropapillary	4 (12.5)	3 (18.8)	1 (6.3)
Solid	12 (37.5)	9 (56.3)	3 (18.8)
Lepidic	6 (18.8)	1 (6.3)	6 (37.5)
Acinar	4 (12.5)	1 (6.3)	2 (12.5)
ND	1 (3.1)	1 (6.3)	–
Baseline tissue origin			
Liver	1 (3.1)	–	1 (6.3)
Lung	24 (75.0)	12 (75.0)	12 (75.0)
Pleura	5 (15.6)	3 (18.8)	2 (12.5)
Lymph node	1 (3.1)	–	1 (6.3)
Small bowel/peritoneum	1 (3.1)	1 (6.3)	–
ECOG			
0	10 (31.3)	1 (6.3)	9 (56.3)
1	19 (59.4)	13 (81.3)	6 (37.5)
2	3 (9.4)	2 (12.5)	1 (6.3)
Number of metastatic sites			
< 2	24 (75%)	9 (56.3)	15 (93.8)
> 3	8 (25%)	7 (43.8)	1 (6.3)
Brain metastases (baseline)			
Yes	10 (31.3)	9 (56.3)	1 (6.3)
No	22 (68.8)	7 (43.8)	15 (93.8)
Lung/pleural metastases			
Yes	28 (87.5)	14 (87.5)	14 (87.5)
No	4 (12.5)	2 (12.5)	2 (12.5)
Liver metastases			
Yes	10 (31.3)	10 (62.5)	–
No	22 (68.8)	6 (37.5)	16 (100.0)
Adrenal metastases			
Yes	3 (9.4)	2 (12.5)	1 (6.3)
No	29 (90.6)	14 (87.5)	15 (93.8)
Bone metastases			
Yes	4 (12.5)	1 (6.3)	3 (18.8)
No	28 (87.5)	15 (93.8)	13 (81.3)

Table 1 (continued)

Variable	Overall population (N = 32)	Cohort A (N = 16)	Cohort B (N = 16)
EGFR mutation			
Exon 19 deletion	15 (46.9)	2 (12.5)	13 (81.3)
L858R	16 (50.0)	13 (81.3)	3 (18.8)
G719S	1 (3.1)	1 (6.3)	–
Secondary EGFR mutations			
Exon 18 p.V869M	1 (3.1)	1 (6.3)	–
Exon 21 p.N826S	1 (3.1)	1 (6.3)	–
Exon 21 p.K846R	1 (3.1)	1 (6.3)	–
Exon 21 p.L861Q	1 (3.1)	–	1 (6.3)
Exon 21 p.G863D	1 (3.1)	1 (6.3)	–
Exon 19 p.D761Y	1 (3.1)	1 (6.3)	–
Exon 19 p.L747S	1 (3.1)	1 (6.3)	–
T790M mutation at baseline			
Yes	5 (15.6)	4 (25.0)	1 (6.3)
No	27 (84.4)	12 (75.0)	15 (93.8)
Number of commutations at baseline			
< 2	18 (56.3)	2 (12.5)	16 (100.0)
> 3	14 (43.8)	14 (87.5)	–
Baseline commutation in P53			
Yes	17 (53.1)	14 (87.5)	3 (18.8)
No	15 (46.9)	2 (12.5)	13 (81.3)
Baseline commutations in RAF pathway			
Yes	6 (18.8)	6 (37.5)	–
No	26 (81.3)	10 (62.5)	16 (100.0)
PD-L1 expression (at baseline)			
<1%	9 (28.1)	3 (18.8)	6 (37.5)
1–49%	21 (65.0)	11 (68.8)	10 (62.5)
> 50%	2 (6.3)	2 (12.5)	–
TMB at baseline			
Mean TMB	4 (±3.0)	1.3 (±0.5)	1.0 (±1.0)
TMB-H	5 (15.6)	5 (31.3)	–
TMB-L	22 (68.8)	11 (68.8)	11 (68.8)
ND	5 (15.6)		5 (31.3)
Post-osimertinib specimens			
Tumor tissue	2 (6.2)	–	2 (12.6)
cfDNA	31 (96.9)	16 (100.0)	15 (93.8)
Pleural effusion	1 (3.1)	–	–

Sociodemographic variables, basal clinic, and molecular characteristics of both cohorts were taken from a centralized database (CLICaP Real World Data/Evidence database)

cfDNA circulating free DNA, CLICaP Latin American Consortium for Investigation of Lung Cancer, ND no data, TMB tumor mutational burden, TMB-H tumor mutation burden–high, TMB-L tumor mutation burden–low, ECOG Eastern Cooperative Oncology Group, NOS Non specified, PD-L1 Programmed Cell Death Ligand 1.

PD-L1 expression was significantly associated with OS, favoring the group of patients with a negative immunophenotype (PD-L1 < 1% 34.1 months, 95% CI 30.6–37.6, vs. PD-L1 1–49% 31.6 months, 95% CI 13.7–24.5, vs. PD-L1 ≥ 50% 19.1 months, 95% CI 13.7–24.5; $P = 0.029$). Low mRNA expression levels for AXL ($P = 0.001$) and high mRNA expression levels for BIM ($P = 0.0001$) and the absence of detectable cfDNA at week 8 of treatment ($P = 0.001$) were also significantly associated with OS (Supplementary Fig. 8A–C). Contrary to the findings for PFS, the presence of baseline T790M did not significantly impact OS ($P = 0.06$; Supplementary Fig. 5B). Univariate analysis showed that the variables that most negatively influenced PFS and OS were the presence of brain metastases
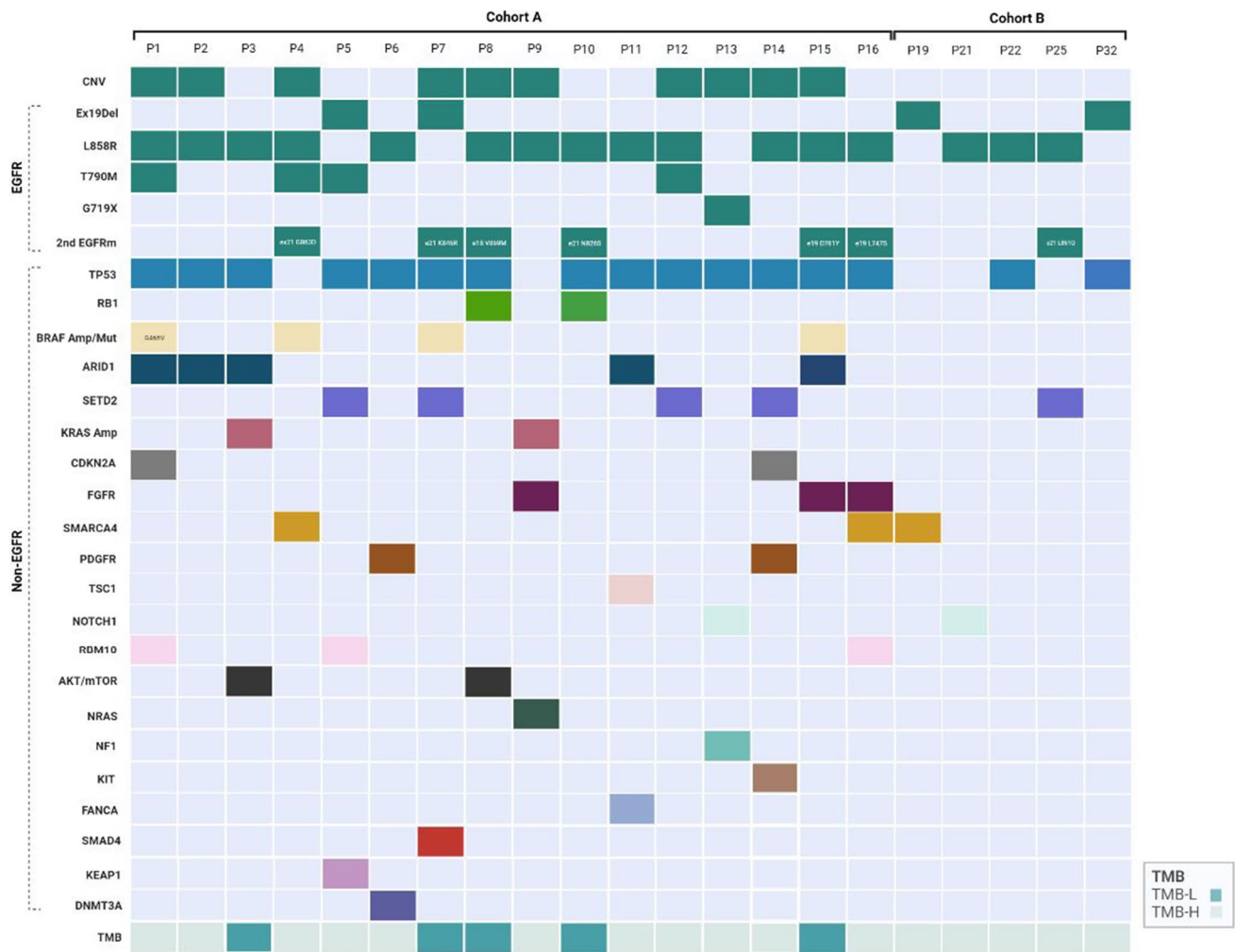
Fig. 2 Genomic characteristics (including copy number variation and TMB) of patients with mutations in cohorts A and B. *EGFR* epidermal growth factor receptor, *TMB* tumor mutational burden, *TMB-H* tumor mutation burden–high, *TMB-L* tumor mutation burden–low

($P = 0.002$), evidence of a more significant number of commutations ($P = 0.001$), TMB-H ($P = 0.0001$), and the high levels of mRNA of *AXL* ($P = 0.01$) and low *BIM* ($P = 0.02$).

3.3 Genomic Alterations Found in Liquid Biopsy After Progression to Osimertinib

Figure 4 summarizes the main alterations in the liquid biopsy after progression to osimertinib (31 patients had a liquid biopsy, and one patient had pleural effusion cell block analysis). Sixty-four genomic alterations were found at the time of progression, 45 in cohort A (70%) and 19 in cohort B (30%). In cohort A, six patients (37.5%) lost the *EGFR*-sensitizing mutation, a condition that did not occur in any patient in cohort B ($P = 0.01$). In addition,

in cohort A, off-target alterations were dominant, mainly in *TP53* (13/35.5%), the *RAS* pathway (13/35.5%), *PI3K* (4/8.8%), and *RB1* (2/4.4%). In contrast, most alterations in cohort B were due to on-target (6/31.2%) or included *MET* amplification (3/15.8%), the *RAS* pathway (2/10.5%), *GNAS* (2/ 10.5%), or *RB1* (2/10.5%). After the second line, two patients had transformation to small cell carcinoma (6.3%), confirmed by biopsy, with mutations in *TP53* and *RB1*. Post-osimertinib mean TMB was 5.0 (SD ± 3.6), with no significant differences between the two cohorts. Supplementary Fig. 9 shows a representative case of primary resistance to osimertinib, including genomic variations throughout the history of the disease (see the electronic supplementary material).
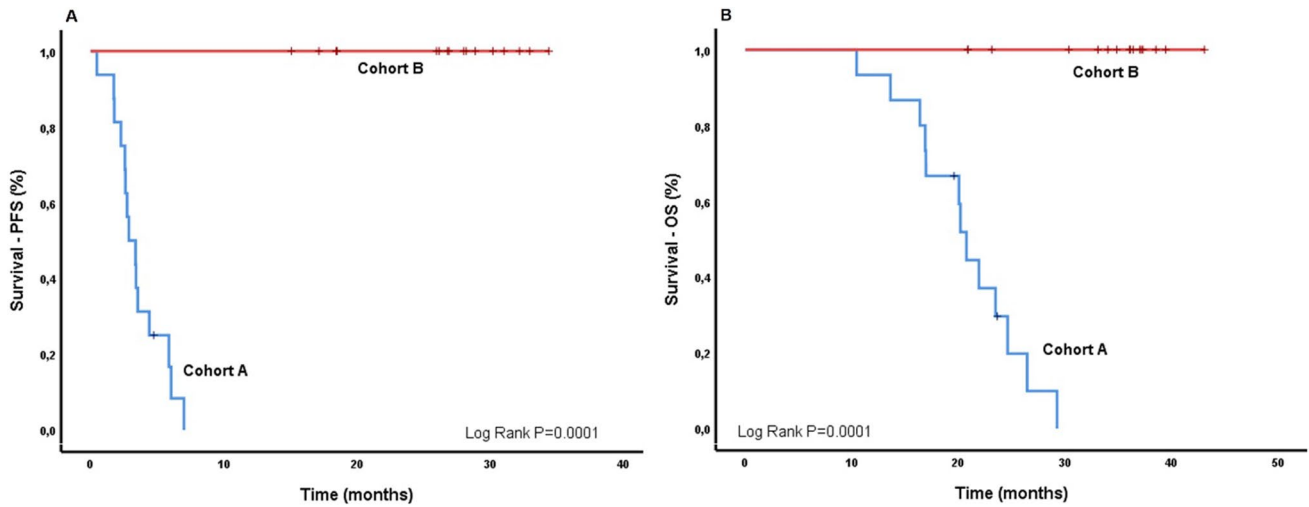
Fig. 3 PFS (**A**) and OS (**B**) for cohorts A and B. PFS with osimertinib was 3.1 months (95% CI 0.47–7.0) for cohort A and NR for cohort B (P = 0.0001) (**A**). After a median follow-up of 25.1 months (95% CI 11.0–43.0), the OS for cohorts A and B was 20.1 months (95% CI 10.4–29.2) and NR (P = 0.0001) (**B**). *CI* confidence interval, *OS* overall survival, *PFS* progression-free survival, *NR* Not reached.

3.4 Second-Line Therapy

Thirty-one patients received second-line treatment (95.6%), mainly with the IMPOWER-150 study schedule (75%) or with the carboplatin/pemetrexed/bevacizumab combination (12.5%). The ORR for the second line was 68.8% (23 cases achieved PR), with no statistically significant difference between the two cohorts. The median PFS obtained with the second line was 8.1 months (95% CI 3.2–12.1) and 11.2 months (95% CI 9.8–12.5; P = 0.22) for cohorts A and B, respectively. The only variable that affected PFS for the second line was PD-L1 expression (PD-L1 < 1% 7.8 months, 95% CI 6.4–8.7, vs. PD-L1 1–49% 9.2 months, 95% CI 8.7–11.2, vs. PD-L1 ≥ 50% 11.6 months, 95% CI 7.3–13.7; P = 0.021).

4 Discussion

The mutational landscape for EGFR-TKI-sensitizing alterations plays a significant role in determining treatment efficacy for patients with NSCLC, and an in-depth
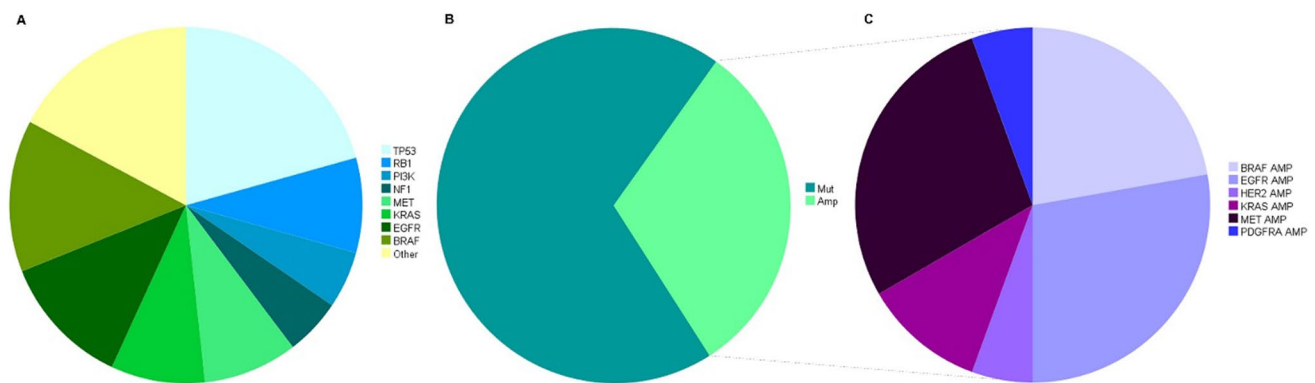
Fig. 4 Genomic alterations were identified at osimertinib progression (liquid biopsy in blood or pleural fluid) considering mutations (**A**) and amplifications (**B**). All patients in cohort A had two or more commutations, while in cohort B, only three had a commutation. **C** Amplifications identified at the time of progression to osimertinib

understanding of this landscape offers an improved therapeutic approach. Nonetheless, the alterations that involve primary resistance to treatment with osimertinib among patients with *EGFR*m NSCLC have not been extensively characterized according to the observed ethnic variation in low-middle-income countries. In this study, we characterized the molecular determinants present in tumors of patients who do not respond to first-line treatment with osimertinib and compared them to patients with durable responses to osimertinib. Our results demonstrated that potential factors associated with poor survival outcomes under osimertinib treatment include the presence of three or more commutations (mainly including the *TP53* gene), high levels of *AXL* mRNA, low levels of *BIM* mRNA, T790M gene de novo presence, TMB-H, and *EGFR* p.L858R variant presence.

Although TKI-acquired resistance has been extensively studied, there are more studies about the molecular interactions that bypass the action of TKI than primary resistance [34]. After exposure to osimertinib, ORR was considerably lower in cohort A (6.3%), with lung involvement as the leading site of progression and two times higher brain involvement. *EGFR*m are associated with the progression of the disease, and the brain is one of the primary sites of metastasis; in fact, patients with T790M usually have a higher brain compromise [35].

TMB-H, high expression levels of *AXL* mRNA, and low mRNA levels of *BIM* were commonly identified in patients with intrinsic resistance. These alterations could add genomic instability burden to the tumor and, in fact, resistance to TKI inhibitors [36, 37]. AXL is a tyrosine kinase receptor that transduces signals from the extracellular matrix into the cytoplasm by binding growth factor GAS6, thus regulating many physiological processes, including cell survival, cell proliferation, migration, and differentiation [38]. High expression of the AXL protein in tumors is reported to be associated with poor prognosis and acquired resistance to targeted therapy in several types of cancer, including glioblastoma, breast cancer, lung cancer, and acute myeloid leukemia [39, 40]. Recently, Taniguchi et al. showed that osimertinib stimulated AXL, which binds to the HER family proteins EGFR and HER3 through a negative feedback loop involving the suppression of sprouty RTK signaling antagonist 4 (SPRY4). In sequence, activated AXL was associated with EGFR and HER3 in maintaining cell survival and inducing the emergence of cells tolerant to osimertinib [36]. Likewise, AXL hyperactivity also includes resistance through EMT closely associated with tumor heterogeneity and induces variations in pathways, including MET, platelet-derived growth factor receptor (PDGFR), and MAPK [39, 41, 42]. In our study, 81% of the cases in cohort A had high

levels of *AXL* mRNA expression, values that were 1.5 times higher than those observed in cohort B.

As mentioned above, low levels of *BIM* mRNA were associated with a worse prognosis. BIM is a BH3-only protein that directly activates the ultimate effectors of apoptosis BAK (BCL-2 antagonist or killer) and BAX (BCL-2-associated X protein) [43]. Recently, Karachaliou and colleagues assessed the role of *BIM* mRNA expression in patients treated in the EURTAC study [44]. The analysis shows that PFS and OS were significantly longer for patients with high *BIM* expression than for those with low/intermediate *BIM* mRNA expression. Patients with low *BIM* expression could derive only a meager benefit from treatment with EGFR-TKI alone. Still, they could benefit from synthetic lethality combinations, including small molecules that mimic the BH3 motif [30]. Previous evidence suggested that gefitinib combined with the BH3 mimetic ABT-737 (an analog of navitoclax) substantially increases apoptosis compared with each agent alone in *EGFR*-mutant H1650 cells with low *BIM* expression [37, 45].

PFS and OS were also significantly lower among patients with primary resistance. In cohort A, patients with ex19del had a higher PFS (and presence of brain metastasis) than those with L858R. Hong et al. had previously described that L858R could have a negative prognostic value in NSCLC patients. In their cohort, patients with mutations in exon 21 had a PFS of almost 8 months [46–48]. In a similar way, in a Korean cohort of patients, ex19del and no brain involvement were associated with a better PFS under TKI treatment [49]. However, for the same cohort, PFS was negatively affected by the presence of T790M, even under treatment with third-line TKI osimertinib. This finding could be explained under the theory of T790M as one possible intrinsic resistance mechanism. However, T790M can also favor other resistance mechanisms, *MET* amplification among them [50]. In our study, cohort A reported a big proportion of basal L858R mutation, whereas cohort B had ex19del predominately. It is essential to address that the presence of ex19del has been associated with better outcomes [3, 8]. However, commutations alter disease progression by modulating the sensitivity to TKI and facilitating more aggressive tumor biology. ex19del alterations are associated with TMB-L impacting OS and not PFS. As Choi et al. demonstrated, the presence of ex19del is associated with a good OS, even under treatment with first-line TKI [51]. However, in our study, patients were treated with a third-generation TKI with better efficacy than other first- or second-generation TKI. In the absence of L858R mutation, the better outcomes under TKI could be related to the description of *EGFR* ex19del as a more sensitizing mutation that causes changes in phosphorylation

and downstream signaling that result in a better response to osimertinib [52, 53].

TMB has been characterized as a possible predictor factor of targeted therapy response. Previous studies have reported the association between TMB-H with outcomes and a shorter time to treatment discontinuation among patients with *EGFR*-mutated NSCLC under TKI treatment. A cohort presented by Offin et al. showed a negative relationship between a higher TMB and OS [54]. Some authors mention that TMB-H can be associated with the sub-clone presence that could favor resistant cell populations under TKI treatment. TMB could be a marker of propensity towards mutagenesis, mainly among *EGFR*-mutant NSCLC [55, 56]. Interestingly, after osimertinib treatment, TMB was not different between the cohorts, suggesting that genomic heterogenicity could be pre-established before selection under TKI.

Regarding the genomic alterations presented after progression to osimertinib, almost two-thirds were identified among patients in the cohort classified as having primary resistance. For this group, some patients lost the *EGFR*-sensitizing mutation. In vitro studies have suggested that this loss could be a possible mechanism of TKI resistance, at least for first- or second-generation TKI [57]. Remarkably, for the cohort with primary resistance, the genomic alterations identified were off-target mutations. *TP53* and *KRAS* pathway *RB1* were the most common. In the context of the *EGFR*-independent resistance mechanism, *TP53* and *RB1* tumor suppressor genes could be identified in almost 50% of LUAD among Caucasian patients [26, 58, 59]. According to the evidence, *TP53* alterations appear during advanced disease, playing a key role in tumor progression more than tumor initiation. *TP53/RB1* mutations in NSCLC are associated with transformation to SCLC under TKI treatment [60, 61]. The two patients who presented phenotypic transformation had *TP53* and *RB1* mutations at the time of biopsy confirmation. *KRAS* is another *EGFR*-independent resistance mechanism that was reported in our study. Although *EGFR* and *KRAS* commutations are rare, *KRAS* amplification could favor resistance through ERBB-family upregulation [20, 62] As expected, in cohort B, *MET* amplification was a common mutation. It is known that under treatment with TKI, *MET* alterations can be found in almost 5% and 20% of patients treated with first- and second-generation EGFR-TKI and osimertinib, respectively [23, 63, 64].

This study, as with other studies focused on specific genomic characterization of primary resistance, has some limitations, such as the small sample size and the specific population that affects external validity. Also, based on the characteristics of the patients, matching both cohorts was not feasible, which might limit the extent of the comparison between cohorts.

5 Conclusions

Understanding the molecular mechanisms that favor primary resistance to EGFR-TKI is fundamental to choosing NSCLC treatment and finding new molecular targets. Targeting *EGFR* could force the selection of tumors with other molecular pathway alterations that allow tumor progression. *EGFR*-independent mechanisms of resistance (or off-target alterations) were found in a population with primary resistance. Treatment with the third-generation TKI osimertinib produces very limited survival outcomes for patients with off-target alterations. These data evidence the need for molecular profiling of patients before the beginning of treatment and the challenge of developing new therapeutic approaches based on specific molecular alterations. As the presence of cfDNA at the time of progression was associated with poor outcomes, liquid biopsy reinforces its role in monitoring the prognosis among NSCLC Hispanic patients.

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Declarations

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