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ORIGINAL ARTICLE

Single-cell DNA-seq depicts clonal evolution of multiple driver alterations in osimertinib-resistant patients

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Background: The development of targeted agents, such as osimertinib for *EGFR*-mutated non-small-cell lung cancer (NSCLC), has drastically improved patient outcome, but tumor resistance eventually always occurs. In osimertinib-resistant NSCLC, the emergence of a second molecular driver alteration (such as *ALK, RET, FGFR3* fusions or *BRAF*, *KRAS* mutations) has been described. Whether those alterations and the activating *EGFR* mutations occur within a single cancer cell or in distinct cell populations is largely debated.

Patients and methods: Tumor sequencing was used to identify the acquired resistance mechanisms to osimertinib in the MATCH-R trial (NCT0251782). We implemented single-cell next-generation sequencing to investigate tumor heterogeneity on patient's frozen tissues in which multiple alterations have been identified. Patient-derived models, cell lines, and patient-derived xenografts were exposed to specific inhibitors to investigate combination treatment strategies.

Results: Among the 45 patients included in MATCH-R who progressed on osimertinib, 9 developed a second targetable alteration ($n = 2$ *FGFR3-TACC3*, $n = 1$ *KIF5B-RET*, $n = 1$ *STRN-ALK* fusions; $n = 2$ $BRAF^{V600E}$, $n = 1$ $KRAS^{G12V}$, $n = 1$ $KRAS^{G12R}$, $n = 1$ $KRAS^{G12D}$ mutations). Single-cell analysis revealed that the two driver alterations coexist within one single cancer cell in the four patients whose frozen samples were fully contributive. A high degree of heterogeneity within samples and sequential acquisitions of molecular events were highlighted. A combination treatment concomitantly targeting the two driver alterations was required on the corresponding patient-derived models to restore cell sensitivity, which was consistent with clinical data showing efficacy of brigatinib in the patient with *ALK* fusion after progression to osimertinib and crizotinib administered sequentially.

Conclusions: Distinct molecular driver alterations at osimertinib resistance coexist with initial *EGFR* mutations in single cancer cells. The clonal evolution of cancer cell populations emphasized their heterogeneity leading to osimertinib relapse. Combining two targeted treatments is effective to achieve clinical benefit.

Key words: single cell, clonal evolution, osimertinib, resistance, double alterations

INTRODUCTION

The development of tyrosine kinase inhibitors (TKIs) has dramatically improved outcomes of patients with oncogene-addicted non-small-cell lung cancer (NSCLC) such as epidermal growth factor receptor (EGFR)-TKIs for the treatment of *EGFR*-activating mutations, which account for 10%-45% of lung adenocarcinomas.[1,2] T790M resistance mutation drives acquired resistance to first- (gefitinib, erlotinib) and second- (afatinib, dacomitinib) generation EGFR-TKIs in ∼50% of cases.[3,4] Osimertinib, the third-generation TKI, selectively inhibits both *EGFR*-activating and secondary T790M mutations. Osimertinib, originally approved for T790M-mediated resistance to early-generation TKIs, has recently moved to the first-line setting, as it demonstrated improved progression-free survival (median 18.9 versus 10.2 months) and overall survival

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(median 38.6 versus 31.8 months) compared with early-generation TKIs, and better activity on central nervous system metastases.[5,6]

Despite high response rates, patients invariably experience disease progression upon osimertinib. Resistance mechanisms to osimertinib are heterogeneous and can be schematically divided between on-target (*EGFR*-mediated resistance, e.g. C797S acquisition) and off-target (bypass signaling pathway such as *MET* amplification, histologic transformation) mechanisms.[7,8]

Emerging data report the acquisition of new driver molecular alterations in addition to the already existing *EGFR*-activating mutation found in tumor cells. These can be either fusions involving *ALK*, *RET*, *MET*, *FGFR*, or *BRAF* and *KRAS* mutations.[9-11] Although such alterations are usually described as mutually exclusive with *EGFR* mutations at diagnosis (0.9% of NSCLC patients in the French Biomarkers France database),[12] recent data have reported their emergence as an osimertinib off-target resistance mechanism. Most importantly, these alterations represent meaningful therapeutic targets, and dual concomitant blockade may effectively overcome resistance.[12,13]

The question of whether those multiple driver alterations occur within a single cancer cell or in distinct cancer cell populations is, however, still pending. We report here the 'in-depth' characterization of osimertinib resistance mechanisms, including the acquisition of targetable driver alterations, and the preclinical results from combinatorial treatment strategies. As clinical observations have demonstrated the benefit of adapting the targeted therapy to acquired alterations at resistance, a clear determination of dual driver alterations at the single-cell level and their clonal evolution leading to tumor heterogeneity could lead to a major impact on clinical practice.

MATERIALS AND METHODS

MATCH-R clinical trial

MATCH-R trial (NCT0251782) is a prospective, institutional study ongoing since 2015 at Gustave Roussy to identify molecular mechanisms of acquired resistance to targeted therapies.[14] Repeated biopsies are collected at diagnosis and relapse, subjected to targeted next-generation sequencing (NGS), whole exome sequencing (WES) and RNA sequencing, and patient-derived models are established to functionally validate the acquired resistance mechanisms. MATCH-R (MR) numbers labeled as '-re' (MR211-re, MR403-re) correspond to patients who had already been biopsied at progression to previous-generation EGFR-TKIs. Translational studies were carried out in the setting of the institutional medical-scientific program 'Unlock' aiming to develop therapeutic strategies to prevent or bypass these resistance mechanisms.

Development of patients-derived xenografts

All animal procedures and studies were carried out in accordance with the approved guidelines for animal experimentation by the ethics committee at University Paris Sud (CEEA 26, Projects 2014-055-2790 for PDX establishment and 2020-074-27871 for pharmacological treatments) following EU regulation.

Fresh tumor fragments from patient MR240 were implanted in the subrenal capsule of 6-week-old female NOD scid gamma (NSG) mice obtained from Charles River Laboratories. PDX were carefully characterized and cancer histology was confirmed by immunohistochemistry (IHC), and oncogenic driver and acquired mutations were validated by panel-targeted NGS Oncomine™ Comprehensive Assay v3M (Thermo Fisher Scientific, Waltham, MA).

Patient-derived cell lines

MR240 cell line was established from the corresponding PDX by enzymatic digestion and mechanic degradation, using the Gentle MACS Dissociator®, Mouse Cell Depletion Kit®, and Auto MACS Pro® (MiltenyiBiotec Inc., Bergisch Gladbach, Germany). Dissociated tumor cells were cultured in 'TCM' media (tumor culture media): Dulbecco's Modified Eagle Medium (DMEM) F12 Glutamax + 10% antibiotic/antimycotic + 10% FBS (fetal bovine serum) + hydrocortisone + adenine + RockInhibitor + 1/10 cholera toxin and plated in T25 flask for cell expansion as previously described.[15] After expansion, cells were plated in T75 flasks with DMEM media (+ 10% antibiotic + 10% FBS) and drugged with 1 μM osimertinib to maintain the drug pressure. Cell lines were incubated at 37°C and humidified air with 5% CO_2. MR393 cell line was established from pleural effusion collected at osimertinib progression. Pleural effusion mononuclear cells were isolated by Ficoll centrifugation and cultured in 'TCM' media as detailed earlier. After stable cancer cell growth obtained *in vitro,* the presence of driver alterations was confirmed in the patient-derived cell lines by RNA extraction, RT-PCR, and DNA Sanger sequencing. The coexistence of both *EGFR* and *ALK* alterations in the MR240 cell line and of *EGFR* and *FGFR3* alterations in the MR393 cell line were validated.

Single nuclei isolation and whole genome amplification from frozen biopsies

Single nuclei sequencing was adapted from Leung et al. (Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2022.01.004).[16] Frozen liquid (MR393 10% tumor cells pleural effusion) and solid biopsies (MR202 30% tumor cells, MR211-re 20% tumor cells, MR240 60% tumor cells, MR385 30% tumor cell) were used to address tumor clonal evolution.

Solid biopsies were cut and minced with a scalpel in Petri dishes and incubated with 1 ml of NST(146nM NaCl, 10mM Tris Base pH7.8, 1mM CaCl2, 21mM MgCl2, 0.05% BSA, and 0.2% Nonidet P-40)-4,6-diamidino-2-phenylindole (DAPI) solution for 90 min. Filtration through a nylon-mesh filter (40 μm) was carried out to remove debris, and nuclei were sorted using fluorescent activated cell sorting. Individual

nuclei were then deposited into a 96-well plate containing 4 µl/well phosphate-buffered saline. Isolation of 50 and 100 cells per well were used as positive controls, and 0 cell as a negative control.

After nuclei lysis, genomic DNA from individual nuclei was amplified by whole genome amplification (WGA) with multiple displacement amplification using Phi 29 polymerase and modified random hexamers, according to the manufacturer's protocol (Repli G human control kit®, Qiagen Venlo, Netherlands). WGA quality control (QC) was carried out by PCR, using a panel of six primer pairs located on six different chromosomes and Fast Start PCR Master Kit assay (Roche Basel, Switzerland). Samples were validated if more than three amplicons could be detected attesting for more than half of the genome being satisfactorily amplified.

WES and data processing

See online-only Supplementary Materials and Methods, available at https://doi.org/10.1016/j.annonc.2022.01.004.

Targeted next-generation sequencing panel on single nuclei

See online-only Supplementary Materials and Methods, available at https://doi.org/10.1016/j.annonc.2022.01.004.

In vitro *cell viability assays*

Cells were treated 24 h after seeding in 96-well plates with 10 serial 1/3 dilutions from a Cmax (maximal concentration) of 30 µM, using three replicates per condition. Combination treatments were carried out using 1 µM anchors for crizotinib and osimertinib, and 300 nM anchors for FGFR inhibitors. Viability assessment was carried out using CellTiterGlo® luminescence assay kit (Promega Madison, WI) and Victor microplate reader. Data were normalized to dimethyl sulfoxide (DMSO) vehicle wells and the half maximal inhibitory concentrations (IC_{50}) were determined using the GraphPad Prism software.

In vivo *pharmacological studies*

MR240 PDX-bearing NSG mice were treated with osimertinib (25 mg/kg qd in HCl 0.1N) or alectinib (25 mg/kg qd in 0.02 N HCl, 10% DMSO, 10% Cremophor EL, 15% PEG400, 15% HPCD) or their combination by oral gavage. Eight mice per group were treated for 40 days and tumor volume and mice weight were measured twice per week.

RESULTS

Osimertinib resistance mechanisms

From January 2015 to October 2020, a total of 45 *EGFR*-mutated NSCLC patients were consecutively included in the prospective MATCH-R trial at osimertinib resistance (Figure 1A). Baseline patients' characteristics are listed in Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2022.01.004. Eight patients received osimertinib as the first-line EGFR-TKI.

WES, targeted NGS, and RNA sequencing of tumor biopsies allowed us to identify putative resistance mechanisms in 82% (37/45) of those patients. Molecular alterations detected were tertiary *EGFR* mutations (mainly C797S) in 29% of cases, bypass pathway activation (mainly *MET* amplification or *KRAS*, *PTEN*, *PIK3CA* mutations) in 53%, and remained unknown for 18% of patients (Figure 1B).

A driver mutational landscape analysis of the 37 available WES was assessed to obtain a more comprehensive analysis of the molecular alterations [single nucleotide variant (SNV) and copy number alteration (CNA)] at osimertinib resistance (Figure 1C). In total, we identified pathogenic alterations in 17 driver genes mutated in more than one patient tumor. A total of 70% of tumors had *TP53* missense mutation and/or loss of heterozygosity suggesting a frequent biallelic inactivation. *EGFR* harbored multiple oncogenic events including recurrent amplification (59%) which might contribute to EGFR activation and osimertinib resistance. Alterations in previously described osimertinib-associated resistant genes (*EGFR C797X, MET, BRAF, PIK3CA, KRAS, NRAS, MAP2K1*) were generally mutually exclusive with each other (full dataset is included in Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2022.01.004).

Acquisition of secondary oncogenic alterations at osimertinib resistance

Within our cohort of 45 patients progressing on osimertinib, in 9 cases (20%) WES and RNA sequencing analysis revealed the acquisition of a secondary oncogenic alteration. These alterations included *KIF5B-RET* (MR48), *STRN-ALK* (MR240), and *FGFR3-TACC3* fusions (MR211-re, MR393), $BRAF^{V600E}$ (MR202, MR218), both $KRAS^{G12V}$ and $BRAF^{G466E}$ (MR403-re), both $KRAS^{G12R}$ and $PI3KCA^{E545K,\ E726K,}$ (MR478), and $KRAS^{G12D}$ (MR385) mutations (Table 1). Tertiary *EGFR* mutations (C797S or C797G) were present in both patients with $BRAF^{V600E}$ mutation. With the exception of MR393 and MR478 who received upfront osimertinib, all patients were previously exposed to first- or second-generation EGFR-TKIs. All samples presented the persistence of *EGFR*-activating mutation at osimertinib progression, although T790M was still detectable in only three samples. Sequencing of matched pre-osimertinib samples did not identify these alterations, suggesting their emergence during treatment. Frozen tissues were available from five out of these nine patients and were selected to perform single-cell isolation (Supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2022.01.004). Blood samples were collected for 21 patients at osimertinib progression to perform circulating tumor DNA (ctDNA) panel sequencing and ddPCR for sensitive detection of EGFR-activating and -resistant mutations. EGFR mutations were detected in 57% (12/21) of the cases and no additional alterations were revealed by ctDNA sequencing (data not shown).

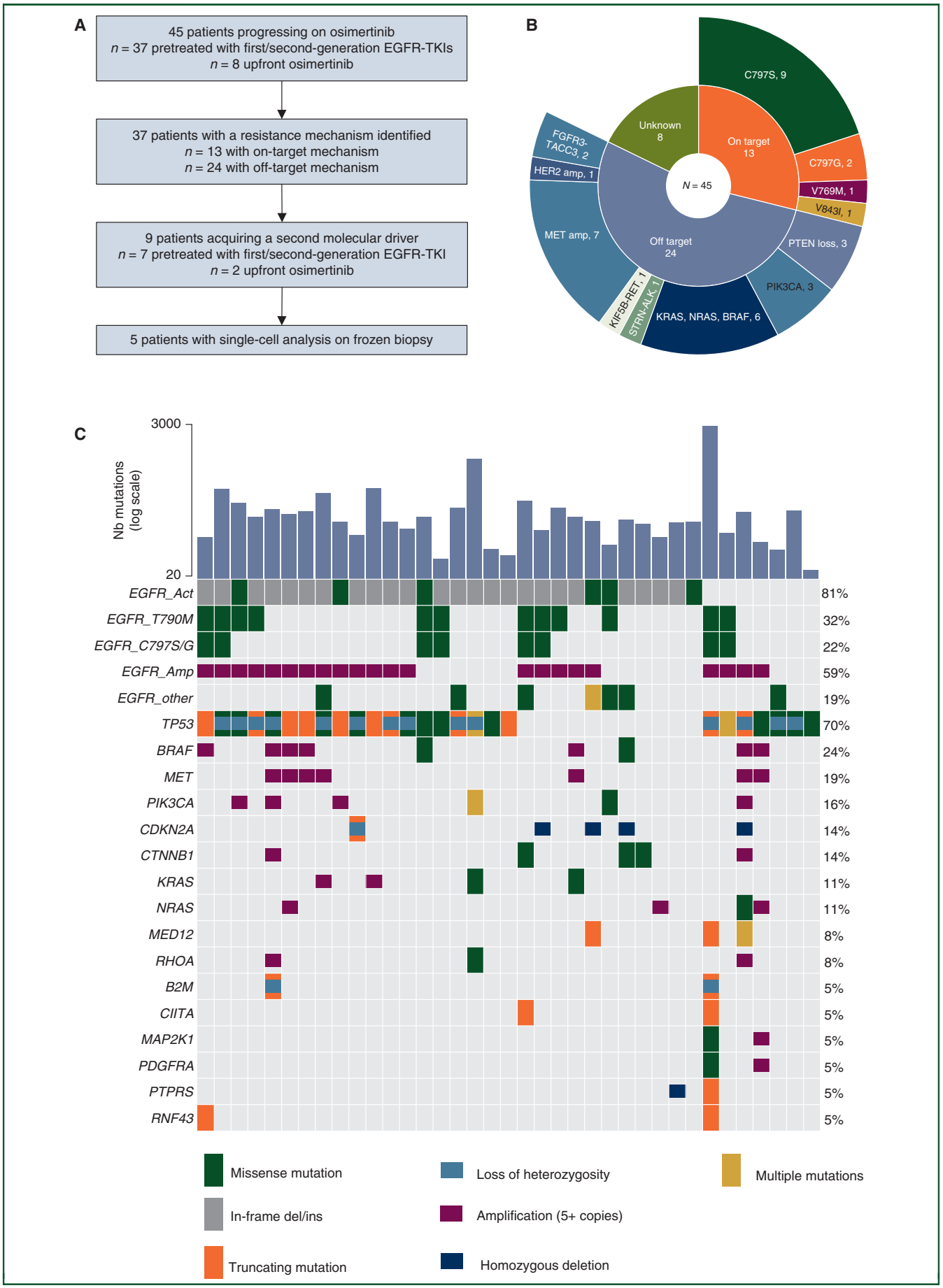
A
45 patients progressing on osimertinib
n = 37 pretreated with first/second-generation EGFR-TKIs
n = 8 upfront osimertinib
37 patients with a resistance mechanism identified
n = 13 with on-target mechanism
n = 24 with off-target mechanism
9 patients acquiring a second molecular driver
n = 7 pretreated with first/second-generation EGFR-TKI
n = 2 upfront osimertinib
5 patients with single-cell analysis on frozen biopsy
B
C797S, 9
C797G, 2
V769M, 1
V843I, 1
PTEN loss, 3
PIK3CA, 3
KRAS, NRAS, BRAF, 6
STRN-ALK, 1
KIF5B-RET, 1
MET amp, 7
HER2 amp, 1
FGFR3-TACC3, 2
Unknown 8
On target 13
Off target 24
N = 45
C
Nb mutations (log scale)
3000
20
EGFR_Act 81%
EGFR_T790M 32%
EGFR_C797S/G 22%
EGFR_Amp 59%
EGFR_other 19%
TP53 70%
BRAF 24%
MET 19%
PIK3CA 16%
CDKN2A 14%
CTNNB1 14%
KRAS 11%
NRAS 11%
MED12 8%
RHOA 8%
B2M 5%
CIITA 5%
MAP2K1 5%
PDGFRA 5%
PTPRS 5%
RNF43 5%
Missense mutation
In-frame del/ins
Truncating mutation
Loss of heterozygosity
Amplification (5+ copies)
Homozygous deletion
Multiple mutations

Table 1. List of nine MATCH-R patients with acquisition of a second driver oncogene at osimertinib resistance

MR identifier	Sex	Age at diagnosis, years	EGFR mutation	Osimertinib first line	TOT (months)	Acquired driver	T790M persistence	C797 status	% Tumor cell in sample	SN analysis
MR48	F	50	ex19del	—	8	KIF5B-RET	—	WT	60	—
MR202	F	65	L858R	—	16	BRAF V600E	+	C797S	50	+
MR211-re	F	57	ex19del	—	14	FGFR3-TACC3	—	WT	30	+
MR218	M	44	ex19del	—	23	BRAF V600E	+	C797G	80	—
MR240	F	66	ex19del	—	21	STRN-ALK	+	WT	60	+
MR385	F	61	ex19del	—	66	KRAS G12D	—	WT	50	+
MR393	F	63	L858R	+	12	FGFR3-TACC3	NA	WT	10	+
MR403-re	F	49	L858R	—	17	KRAS G12V BRAF G466E	—	WT	10	—
MR478	F	86	ex19del	+	24	KRAS G12R PIK3CA E545K PIK3CA E726K	NA	WT	80	—

EGFR, epidermal growth factor receptor; SN, single nuclei; TOT, time on treatment; WT, wild type.

Characterization of genomic STRN-ALK *and* FGFR3-TACC3 *fusion breakpoints*

To evaluate the presence of genomic fusions within single cancer cells, a prerequisite was the identification of the *STRN-ALK* (MR240) and *FGFR3-TACC3* (MR393, MR211-re) fusion breakpoints on genomic DNA.

STRN-ALK rearrangement involves STRN coiled-coil domain and ALK kinase domain on chromosome 2. As the *STRN-ALK* intronic breaking site in genomic DNA was previously unknown, we designed a library of primer pairs involving intron 3 of *STRN* and intron 19 of *ALK* (12653 base pairs). The intronic fusion breakpoint between *STRN* and *ALK* was successfully identified in MR240 genome corresponding to a loss of 6964 intronic base pairs (Supplementary Figure S3A, available at https://doi.org/10.1016/j.annonc.2022.01.004).

FGFR3-TACC3 rearrangement involves FGFR3 kinase domain and TACC3 coiled-coil domain on chromosome 4. The same procedures were carried out to highlight the genomic breakpoint (within intron 16 of *FGFR3* and intron 8 of *TACC3*) which was identified leading to a deletion of 276 and 93 base pairs in MR393 and MR211-re, respectively (Supplementary Figure S3B and C, available at https://doi.org/10.1016/j.annonc.2022.01.004).

Identification of dual driver alterations at the single-cell level

Tissue dissociation and single nuclei sorting in 96-well plates were carried out from frozen patient biopsies (MR240, MR211-re, MR202, and MR385) or pleural effusion (MR393). WGA was carried out on each isolated nuclei and amplified genomic DNA was successfully validated by QC PCR for a total of 281 single nuclei (Supplementary Figure S4, available at https://doi.org/10.1016/j.annonc.2022.01.004). The presence of *EGFR* driver alteration [i.e. *EGFR* exon 19 deletions ($EGFR^{ex19del}$) and L858R mutation on exon 21 ($EGFR^{L858R}$)] was investigated by PCR and Sanger sequencing on 254 validated single nuclei from MR240 (analyzed nuclei $n = 40$), MR211-re ($n = 82$), MR202 ($n = 38$), MR385 ($n = 36$), and MR393 ($n = 58$). The same procedures were carried out to identify the presence of *BRAF, KRAS* mutations or *STRN-ALK, FGFR3-TACC3* fusions.

The coexistence of both, an activating *EGFR* mutation and a new oncogenic alteration, was confirmed in 106 nuclei, corresponding to MR211-re (*EGFR/FGFR3*; $n = 43$, 52%), MR240 (*EGFR/ALK*; $n = 37$, 93%), MR385 (*EGFR/KRAS*; $n = 5$, 14%), and MR393 (*EGFR/FGFR3*; $n = 21$, 37%) (Table 2). In MR202 (*EGFR/BRAF*), *EGFR* mutation was identified in 18 nuclei (47%), but the existence of $BRAF^{V600E}$ mutation was not detected in any nucleus. Altogether these data suggested a coexistence of two targetable oncogenic drivers in all contributive cases.

Clonal evolution at osimertinib resistance

In order to address the tumor heterogeneity at osimertinib resistance, single nuclei were subjected to the tumor mutation load (TML)-targeted NGS assay. Interestingly, neighbor-joining analysis, based on the reliable mutations identified by WES and then detected in targeted NGS, revealed a significant extent of heterogeneity within the patient biopsies, represented by the tree architectures of 7 to 15 branches grouping the cell populations (Figure 2). The lack of WES data for MR393 impaired the generation of neighbor-joining analysis and was not included in this analysis.

These data also exhibit the sequential acquisition of genomic alterations. Normal cells, without somatic mutations, were grouped first and then cell populations harboring the *EGFR*-activating mutation were defined before reaching more mutated cell populations leading to the acquisition of the second oncogenic driver. MR211-re and MR240 mainly contained resistant cancer cells

Figure 1. Osimertinib resistance mechanisms.
(A) Study flowchart of the patients included. (B) On-target and off-target resistance mechanisms identified in 45 osimertinib-resistant patients. (C) Oncoplot mutational landscape of 37 patients' tumor at osimertinib relapse from WES analysis. Somatic SNV and CNA identified in more than one patient are presented. Percentage frequency for each gene is shown on the right and the total number of somatic mutations in each sample is indicated at the top.
CNA, copy number alteration; EGFR, epidermal growth factor receptor; SNV, single nucleotide variant; TKI, tyrosine kinase inhibitor; WES, whole exome sequencing.

Table 2. Coexistence of both primary EGFR and a new oncogenic alteration ('Driver 2') by single nuclei sequencing

	EGFR/Driver 2, n (%)				n (%)
	+/+	+/−	−/+	−/−	Total
MR202	0	18 (47)	0	20 (53)	38 (100)
MR211-re	43 (52)	15 (18)	13 (16)	11 (13)	82 (100)
MR240	37 (93)	1 (3)	0	2 (5)	40 (100)
MR385	5 (14)	6 (17)	0	25 (69)	36 (100)
MR393	21 (37)	15 (27)	5 (9)	17 (29)	58 (100)

EGFR, epidermal growth factor receptor.

expressing the two targetable oncogenic drivers with very slight heterogeneity for MR211-re opposed to six different populations for MR240 (Figure 2D and F).

As depicted in the graphical representation of the results, in two cases the resistant cell population with dual driver originated from T790M-negative cells still present at progression to first-generation EGFR-TKI (MR211-re and MR385 - Figure 2C and G), whereas in the two others cases, the resistant cell population originated from T790M-positive cells (MR240 and MR202—Figure 2A and E). Interestingly, in the MR385 sample, no single trace of T790M-mutated cell was detected at osimertinib progression (Figure 2G and H). These data highlight the large extent of tumor heterogeneity within cancer samples as this analysis was carried out on tiny needle biopsies not integrating spatial and temporal factors.

Of note, MR211-re bulk WES failed to detect any T790M-positive cell at osimertinib relapse, but two single nuclei were detected as being T790M mutated (Figure 2D). This illustrates the benefit of performing single-cell analysis of tumor samples to gain more details on subclonal genomic events that would not be captured in bulk analysis.

Treatment strategies to overcome resistance

Patient-derived cell lines were established from MR240 and MR393 patient samples and were used to perform dose-response viability assays (IC_{50}). MR393 cell line expressing $EGFR^{L858R}$ and FGFR3-TACC3 fusion protein was exposed to incremental doses of osimertinib and FGFR-TKIs. Combining osimertinib with FGFR inhibitors (futibatinib or erdafitinib) allowed to restore the sensitivity of MR393 cell line (Figure 3A and B and Supplementary Figure S5, available at https://doi.org/10.1016/j.annonc.2022.01.004). Similarly, in MR240 cell line, expressing $EGFR^{ex19del}$ and STRN-ALK fusion protein, the sensitivity to osimertinib was restored by the addition of crizotinib (Figure 3C).

To further validate these overcoming strategies, MR240 PDX was treated *in vivo* by alectinib and osimertinib alone or in combination. Consistent with our *in vitro* data, only the combined administration of both TKIs was able to induce tumor regression in mice including a complete response in half of the animals (Figure 3D). MR240 patient was treated by crizotinib alone after osimertinib-acquired resistance. However, inhibiting the ALK fusion protein only was not sufficient to induce a clinical response as the patient tumor was primary resistance to crizotinib. The patient subsequently received the dual ALK/EGFR inhibitor brigatinib,[17] and experienced a sustained clinical response with a 6-month disease stabilization (SD) (Figure 3D).

Altogether, these data confirm the necessity of targeting simultaneously the two oncogenic drivers in order to restore tumor sensitivity when a bypass mechanism is acquired within single cancer cells at resistance.

DISCUSSION

Mechanisms of acquired resistance to osimertinib are under active investigation both in the second-line (T790M-positive),[18-20] and first-line setting.[21] As acquired resistance seems inevitable, physicians are now facing the challenge of next-line therapy selection. This study contributes to understand the adaptive mechanisms underlying resistance to osimertinib, by focusing on the acquisition of new driver alterations upon osimertinib administered either as upfront treatment or at progression to first/second-generation TKIs.

Our mutational landscape analysis revealed a relatively limited number of genes altered at osimertinib resistance (17). Consistent with previous reports, acquired alterations were EGFR C797X (24%), MET amplification (19%), or SNV in genes involved in MAPK or PI3K pathways.

In line with the literature and unprecedented with other TKIs, a significant number of our patients acquired oncogenic fusions at osimertinib progression. Importantly, such fusions are known to represent actionable targets that can be effectively inhibited by specific TKIs. Objective responses have indeed been reported by combining EGFR (osimertinib) and RET (selpercatinib) TKIs in patients with *RET* rearrangement at osimertinib progression.[13,22] Acquired *ALK* fusions have also been described at osimertinib progression, with a clinical benefit of EGFR- and ALK-TKIs combination.[23,24] Similarly, a partial response was achieved by combining FGFR and EGFR-TKIs in a patient with *FGFR3-TACC3*—mediated resistance to osimertinib.[25] *BRAF* and *KRAS* oncogenic mutations have been identified in our study as well. Acquired $BRAF^{V600E}$ mutation has been reported at osimertinib progression in few cases and represents a meaningful therapeutic target in several tumor types.[9] Our study revealed the emergence of *KRAS* G12D, G12V, and G12R mutations upon osimertinib treatment. *KRAS* recently became a targetable oncogene with G12C mutation,[26] and the upcoming development of specific inhibitors for additional *KRAS* mutations can be expected.[27] This study confirms that molecular analyses and systematic rebiopsy programs should be considered at osimertinib progression and that oncogenic fusions should be looked for closely, and included in molecular analyses by NGS and RNA-based fusion panels.

While most of these acquired molecular alteration had been described upon osimertinib progression in various series, the question of whether the two driver alterations can coexist within a single tumor cell has not been solved so far. NGS data often give an average overview of multiple cell populations but cannot accurately resolve the complex heterogeneity of cancer samples. Single-cell sequencing has

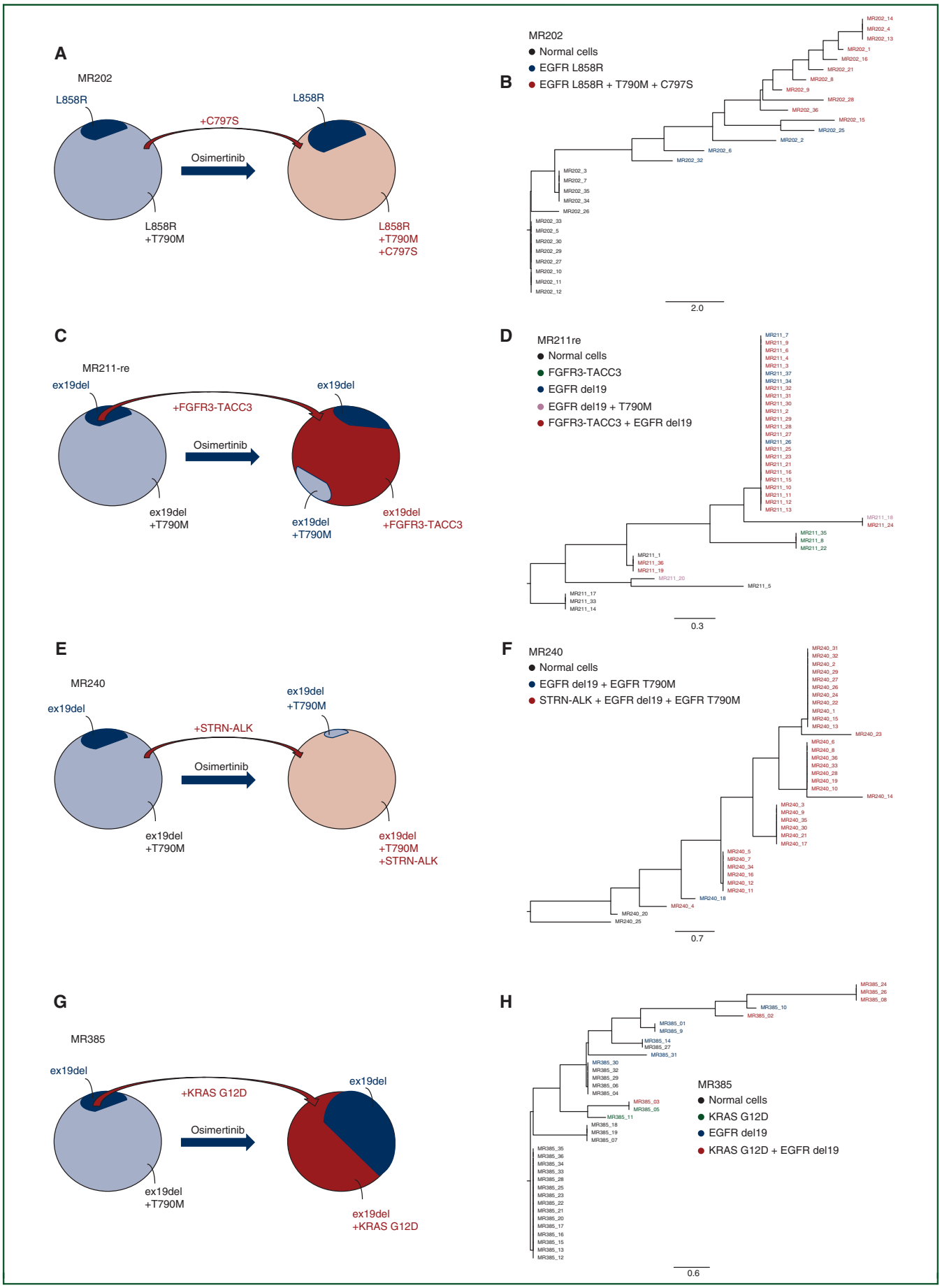

Figure 2. Clonal evolution at osimertinib resistance.

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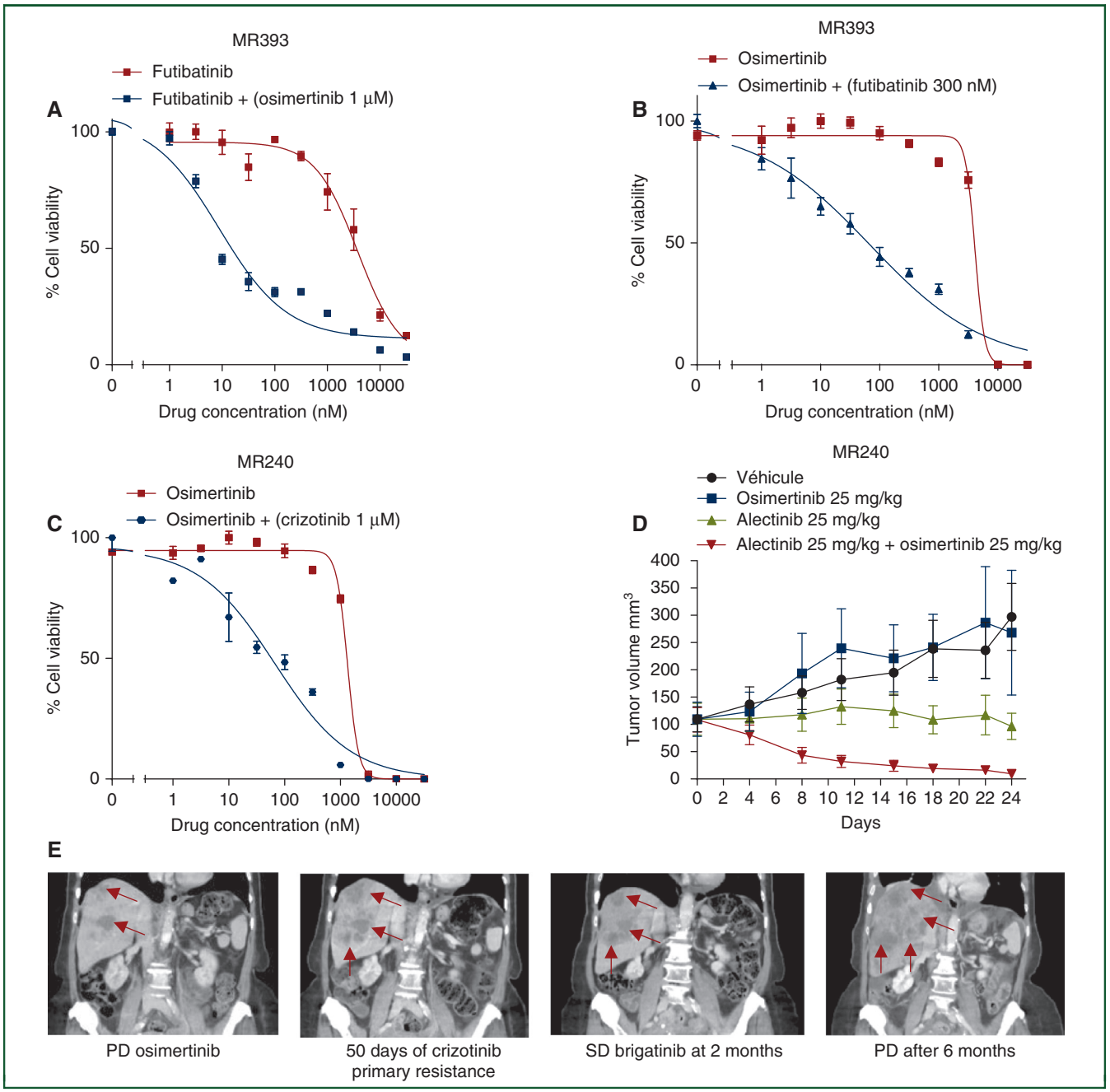

Figure 3. Overcoming osimertinib-acquired resistance by TKI combination.
(A, B) Cell Titer Glo cell survival assessment of MR393 patient-derived cell line established from patient's pleural effusion. Cells were treated with incremental concentrations of osimertinib and futibatinib alone or in combination (A, 1 μM anchor osimertinib; B, 300 nM anchor futibatinib). (C) Cell survival assessment of MR240 patient-derived cell line treated with incremental concentrations of osimertinib alone or in combination with 1 μM anchor crizotinib. Cell viability was assessed at 72 h. Data are shown as a percentage of vehicle-treated control and are the mean ± SEM (standard error of the mean) of three independent biological replicates. (D) *In vivo* pharmacological evaluation of combined osimertinib and alectinib TKIs in MR240 PDX model. Tumor volumes mean ± SEM ($n = 8$). (E) Thoracic computed tomography images of patient MR240, after osimertinib-acquired resistance, during crizotinib or brigatinib treatments. Tumor growth observed during crizotinib treatment is consistent with the necessity of dual oncogene inhibition achieved by brigatinib.
PD, progressive disease; PDX, patients-derived xenografts; SD, stable disease.

thus been developed and constitutes a unique approach to study the intratumoral heterogeneity and characterize the cancer cells clonal evolution in greater details.[16] Although it is believed that such oncogenic alterations are mutually exclusive with primary *EGFR* alteration, we demonstrated that two driver alterations do coexist within one single tumor cell. Indeed, the coexistence of both activating *EGFR* alteration (ex19del or L858R mutation) and acquired fusions

(A-D) Graphical representation of main cellular populations revealed by single nuclei sequencing. (E-H) Each patient's clonal architecture is presented by a tree whose nodes represent clonal evolution; branches represent evolution paths (length scaled by the square root of the number of clonal mutations). Individual nuclei are colored according to driver mutations.

(*FGFR3*, *ALK*) or mutations (*KRAS*) was identified in 106 single nuclei isolated from frozen biopsies (MR211-re, MR240, MR385) or pleural effusion (MR393) at osimertinib progression. The coexistence of $BRAF^{V600E}$ and $EGFR^{L858R}$ mutations could not be evaluated in MR202 biopsy as no single $BRAF^{V600E}$ was detected in our single-cell analysis. This might be explained by the low frequency of the *BRAF* mutation (6%) compared with the high abundance of EGFR mutation (63%) in the patient sample. These results constitute an important contribution to the understanding of tumor adaptation to targeted therapy, as to our knowledge this is the first report of precise detection of dual alterations within single cancer cells from solid lung tumors.

The clear determination of dual driver alterations at the single-cell level has major impact on clinical practice. Although experience in treating bypass alterations remains scarce, evidence has emerged suggesting the clinical benefit of targeting these mechanisms of resistance. In our study, following the MATCH-R biopsy, the patient MR240 experienced a prolonged disease stabilization with the dual ALK/EGFR inhibitor brigatinib. Our *in vitro* experiments on MR393 (*EGFR*+*FGFR3-TACC3* alterations) and MR240 (*EGFR*+*STRN-ALK* alterations) patient-derived cell lines suggest that combining osimertinib with FGFR or ALK inhibitors allows to restore sensitivity by inducing a synergistic cytotoxic effect. Similarly, pharmacological evaluation on the corresponding MR240 PDX demonstrated the mandatory use of a simultaneous treatment to restore cancer cell sensitivity. Revealing the coexistence of a double driver within a single cell rationalizes this observation as it is easy to understand that one driver would compensate for the inhibition of the other when single-agent treatment is clinically evaluated.

Facing potential toxicity profiles, it is possible that clinicians and regulatory agencies favor a sequential administration of pharmacological agents rather than simultaneous treatment. However, given the results of this study, as the two driver alterations exist within a single cell, combination strategies should be considered over a sequential approach. These data on mechanisms of acquired resistance after osimertinib strongly support the concept of the ORCHARD trial exploring drug combination treatments post progression that needs to be intensified by evaluating additional combinations in clinical practice.[28]

One limitation of our study is the limited number of patients' material available for single-cell sequencing. Five out of the nine samples identified could be correctly analyzed, and the coexistence of the two driver alterations could be evaluated in four of them. Within the first-line osimertinib population, median time on treatment (TOT, 11.5 months) was inferior compared to patients who received osimertinib after first/second-generation EGFR-TKI (median TOT 18.0 months). This was likely attributable to a selective bias due to the recent introduction of first-line osimertinib in clinical practice leading to the intrinsic selection of patients who progressed to upfront osimertinib with a relatively short TOT. Our *in vitro* and *in vivo* data remain limited, as only one PDX and two patient-derived cell lines could be generated for pharmacological analyses, and further studies are needed to confirm the benefit of combinations strategies over sequential approaches. For 18% of the patients, no genetic alteration explaining the resistance to osimertinib was detected. Resistance mechanisms might be linked to previously described adaptations such as phenotypic changes, epigenetic modifications or apoptotic defects.[29] Further efforts are needed to reveal other cancer cell adaptive mechanisms on targeted therapies, which would guide additional therapeutic strategies in clinical practice. ctDNA analysis is usually essential to exhaustively capture molecular heterogeneity at resistance. As the objective of our study was to provide evidence at the single-cell level, we decided to focus our heterogeneity studies on tumor biopsies. Finally, we cannot formally exclude that the second oncogenic drivers might have been preexisting in a very small cell population that was missed in the pre-osimertinib samples.

In summary, this study confirms the acquisition of a new driver alteration as a mechanism of resistance to osimertinib in *EGFR*-mutated lung cancer, and that repeated tumor samplings, together with patient-derived models, can provide new insights into tumor clonal evolution and mechanisms leading to disease progression. Highlighting the coexistence of multiple oncogenic alterations within one single tumor cell at osimertinib progression helps us to understand mechanisms underlying cell adaptation to oncogene-driven tumor inhibition and enables to consider novel therapeutic strategies with significant impact on clinical practice.

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DISCLOSURE

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