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Ultra-sensitive *EGFR*^{T790M} Detection as an Independent Prognostic Marker for Lung Cancer Patients Harboring *EGFR*^{del19} Mutations and Treated with First-generation TKIs

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Abstract

Purpose: The detection of preexisting *EGFR*^{T790M} subclones and the assessment of their clinical significance in the pretreatment of patients with *EGFR*^{T790M} non–small cell lung cancer (NSCLC) remain unclear.

Experimental Design: A total of 179 tumor samples from patients treated or not with a first-generation tyrosine kinase inhibitor (TKI) was analyzed. The presence of ultra-low levels of preexisting EGFR^{T790M} mutation was evaluated using ultra-sensitive droplet digital PCR (ddPCR) and the clinical implication of these mutations on first-generation TKI efficiency assessed.

Results: With a ddPCR linear performance of 0.999 and an analytical sensitivity of approximately 0.001%, we observed a 66% (99/150) overall incidence of ultra-low *EGFR*^{T790M} mutation. Among 82 patients harboring *EGFR*^{activating} mutations, the presence of a preexisting *EGFR*^{T790M} mutation prior to any treatment was significantly associated with a longer progression-free survival (PFS; $P = 0.009$; log-rank test). Interestingly, longer PFS was linked to concomitant *EGFR*^{del19} and ultra-low *EGFR*^{T790M} mutations. Moreover, the presence of both *EGFR*^{del19} and ultra-low *EGFR*^{T790M} mutations was identified as the best fit for predicting the clinical outcome of patients treated with TKI compared with an ultra-low *EGFR*^{T790M} mutation status or an activating mutation alone ($P = 0.042$ and $P = 0.0071$, respectively).

Conclusions: We demonstrate that the detection of the ultra-low *EGFR*^{T790M} mutation in TKI-naïve patients is not a rare event. We suggest that ddPCR should be used in clinical practice to distinguish patients who may respond to first- or third-generation TKIs.

Introduction

The first generation of EGFR tyrosine kinase inhibitors (TKI), including gefitinib and erlotinib, were able to effectively treat advanced patients with non–small cell lung cancer (NSCLC) harboring activating mutations in the EGFR-TK domain (1). Most of these mutations consists in either a small inframe deletions in exon 19 (*EGFR*^{del19}) or a missense mutation in the exon 21 at position 858 (*EGFR*^{L858R}). Unfortunately, the response to first-generation EGFR-TKIs is severely impaired in almost all patients by the unavoidable emergence of resistance to targeted therapy within 12 to 18 months (2–4). Among the different identified mechanisms of acquired resistance identified, the secondary missense T790M mutation (*EGFR*^{T790M}) in *cis* with a primary activating mutant *EGFR* (*EGFR*^{activating}) allele has been reported in approximately half of all cases (5–7).

Increasing evidences suggest that the development of resistant clones may follow temporal patterns through the emergence of preexisting resistant *EGFR*^{T790M} clones and *de novo* acquisition of the *EGFR*^{T790M} mutation in initially *EGFR*^{T790M}-negative drug-tolerant cells (8–10). Under the selective pressure of EGFR TKI therapy, *EGFR*^{T790M} subclones grow until they predominate over *EGFR*^{del19} or *EGFR*^{L858R} mutation cells in the tumor mass (11). Interestingly, the importance of early detection of the *EGFR*^{T790M} mutation, even at very low levels, is supported by the fact that progression-free survival (PFS) in response to EGFR-TKI is reduced in patients harboring the *EGFR*^{T790M} mutation compared with those not carrying the mutation (11–14). This observation is strengthened by two meta-analyses that suggest that the pretreatment *EGFR*^{T790M} mutation has the potential to predict the effect of EGFR-TKIs in *EGFR*^{activating}-positive patients (15, 16). Finally,

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Translational Relevance

Patients with non–small cell lung cancer (NSCLC) that harbored activating mutations in the tyrosine kinase (TK) domain of the EGFR benefit from the administration of TK inhibitors (TKI). However, resistance to targeted therapy unavoidably emerge with, in 50% of the cases, presence of a T790M mutation (*EGFR*^{T790M}). At present, the clinical impact of the presence of a small proportion of clones harboring the *EGFR*^{T790M} mutation in a heterogeneous tumor before EGFR TKI treatment remains controversial. Here, we observed that in TKI-naïve patients, the presence of ultra-low *EGFR*^{T790M} detected by highly sensitive droplet digital PCR (ddPCR) method predicts a better outcome of patients treated with TKI. More particularly, patients harboring a concomitant *EGFR*^{del19} and ultra-low *EGFR*^{T790M} mutations exhibited higher progression-free survival (PFS). These findings may help clinicians to select patients at diagnosis who are likely to develop *EGFR*^{T790M} after treatment and adapt treatments to improve patient management.

third-generation EGFR inhibitors, such as rociletinib or osimertinib, have emerged as potential therapeutics to block the growth of *EGFR*^{T790M}-positive tumors (17). Indeed, osimertinib was recently shown to be more efficient than conventional EGFR-TKIs and is being approved as a first-line treatment (18). Nonetheless, it remains unclear whether the benefit observed with osimertinib is due to the inhibition of the intrinsic *EGFR*^{T790M} cell growth or to a delay of its acquisition.

Detection of preexisting *EGFR*^{T790M} mutations in patients with NSCLC by conventional approaches, such as direct sequencing, typically reveals that 1% to 8% of them harbor the mutation (7, 19, 20). However, the mutation prevalence is clearly underestimated because of the sensitivity of the techniques employed. More sensitive approaches, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), allele-specific PCR, Scorpion Amplification Refractory Mutation System (SARMS), or next-generation sequencing (NGS), have resulted in higher but widely different incidences of pretreatment *EGFR*^{T790M}, depending on the technique used (7, 13, 14, 21, 22). Although these techniques exhibit higher sensitivity, they do not allow ultra-low detection of the *EGFR*^{T790M} mutation, which may be required to tailor personalized therapies for patients with NSCLC.

Droplet digital PCR (ddPCR) is a recently developed technology that enables accurate quantification of very low amounts of target nucleic acid sequences in a sample. In brief, ddPCR measures absolute quantities by counting nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions that are subsequently amplified by PCR and independently analyzed. On the basis of Poisson distribution, it is assumed that small-volume reaction compartments must contain 0 or 1 DNA molecule(s). The absolute concentration of the target is determined after end-point PCR quantification of positive compartments. Therefore, droplets containing mutated or nonmutated DNA strands can be discriminated by flow cytometry, allowing ultrasensitive detection of mutated alleles in a vast background of wild-type (WT) alleles.

Here, we report the use of a highly sensitive ddPCR method to detect ultra-low levels of the *EGFR*^{T790M} mutation and its corresponding WT allele in samples from a large cohort of 179 surgically resected patients with NSCLC to determine the frequency of this molecular alteration and to assess its correlation with clinical outcome.

Materials and Methods

Patients and sample collection

From 2012 to 2015, 2640 patients with a primary treatment-naïve NSCLC tumor underwent mutation detection analysis at the University Hospital of Montpellier (Montpellier, France) and Toulouse University Hospital (Toulouse, France). Of these patients, tissue was available for 179 patients. This study was performed with approval from the Institutional Review Board and in concordance with regulatory guidelines regarding assay validation. For this noninterventional study, an approved informed consent statement was acquired for all patients. All corresponding lesions were excised and submitted for standard pathologic examination. The percentage of tumor cells in the series ranged from 10% to 100% (Supplementary Table S1). Tissue punches using a 1-mm needle or macrodissected 10-μm thick section were performed from tumor paraffin blocks to increase the percentage of tumor cells in the sample. Patients treated with TKI exclusively received a first-generation TKI (gefitinib or erlotinib). Medical records were reviewed to extract clinicopathologic data, including sex, age, smoking status, diagnoses, therapeutic agents, and survival. Tumor progression was defined according to RECIST 1.1 criteria (23).

DNA extraction and mutation testing

DNA extraction was achieved using the QIAamp DNA FFPE Tissue Kit (Qiagen) or Maxwell FFPE Plus LEV DNA Purification Kit (Promega) according to the manufacturer's recommendations. Extracted DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). *EGFR* (exons 18, 19, 20, and 21), *KRAS* (exons 2 and 3), *BRAF* (exon 15), and *HER2* (exon 20) were screened using high-resolution melting (HRM) analysis. Samples with a deviation compared with the WT control samples were further analyzed by Sanger sequencing, as described previously (24).

ddPCR

The presence of the *EGFR*^{T790M} mutation was assessed using QX100 ddPCR System (Bio-Rad). Forward and reverse gene-specific primers and fluorescent hydrolysis probes specific for either the mutant (T790M-FAM) or WT (WT T790-HEX) sequence at codon 790 were obtained from Bio-Rad. Briefly, 50 ng of DNA was used per ddPCR reaction with ddPCR Supermix (Bio-Rad). The reactions were then emulsified using a QX100 droplet generator, transferred to 96-well plates and amplified using the following cycling conditions: 95°C for 10 minutes; 40 cycles of 94°C for 30 seconds, and 55°C for 1 minute; and 98°C for 10 minutes. After amplification, plates were read and fluorescence signal of individual sample droplets were analyzed with a QX100 droplet reader. The mutant allele frequency relative to that of WT was determined using QuantaSoft V.1.7.4 software by applying a correction based on the Poisson distribution to the number of droplets positive for either mutant or WT DNA. Samples with at least two *EGFR*^{T790M}-positive droplets or greater than 2,000

WT-positive droplets detected by ddPCR were included in our study. Samples with less than two $EGFR^{T790M}$-positive droplets detected need to have a minimum of 2,000 WT-positive droplets counted to be retained (Supplementary Fig. S1). These criteria allow a minimum limit of detection of 0.1%.

Statistical analysis

The relationship between $EGFR^{T790M}$ allele frequency and the patients' clinicopathologic characteristics was analyzed using the χ^2 test or the Fisher test depending on the number of the patients per group. PFS and overall survival (OS) were estimated using the Kaplan–Meier method, and the significance of differences between survival rates was ascertained with the log-rank test using SPSS Software (SPSS Inc.). Candidate prognostic factors for PFS with a 0.1 significance level in univariate analysis were entered in a multivariate Cox model, and a backward selection procedure was used to determine independent prognostic markers. A likelihood ratio test was applied to select the best fit between models. $P < 0.05$ was considered significant.

Results

Assessment of ddPCR assay sensitivity for ultra-low $EGFR^{T790M}$ mutation detection

To assess the sensitivity of our ddPCR assay, we first analyzed gDNA reference standard control samples that each harbored a precise $EGFR^{T790M}$ mutation allelic frequency (i.e., 5%, 1%, 0.1%, 0.05%, 0.005%, and no mutation). As shown in Supplementary Fig. S2A, a high and significant correlation between the expected and observed variant frequencies was observed ($R^2 = 0.9998$; $r = 0.9999$; Pearson correlation test; $P < 10^{-4}$). To determine the limit of detection of our assay, we analyzed the limit of the blank defined by the frequency of positive droplets measured in controls without DNA ($N = 48$), formalin-fixed, paraffin-embedded (FFPE) normal tissue samples ($N = 10$) and FFPE tumor samples from other types with mutational profiles similar to those observed lung cancer ($N = 30$; 10 colorectal carcinomas with or without *KRAS* mutation, 10 gastrointestinal stromal tumors with or without *KIT* mutation, and 10 melanomas with or without *BRAF* mutation; Supplementary Table S2). No false-positive droplet events were detected in the negative controls. Regarding the tissue samples, one false-positive droplet was detected in two normal samples and in three tumor samples. According to these results, tumor samples were considered positive when at least two positive droplets were measured.

We next used two FFPE samples previously characterized by NGS or ddPCR and reported to harbor an $EGFR^{T790M}$ mutation at a variant allele frequency (VAF) of 26% or 1%, respectively. Several dilutions of these samples were assessed by ddPCR to evaluate the reproducibility of detection according to the quantity of input DNA. Our results revealed that the $EGFR^{T790M}$ mutation was detected at an identical VAF, regardless of the quantity used (from 50 ng to 0.5 ng) (Supplementary Fig. S2B and S2C).

Patient cohort

A total of 179 tumor samples were analyzed by ddPCR to evaluate the presence of preexisting $EGFR^{T790M}$ mutations. All samples were adenocarcinomas obtained prior to the initiation of any treatment; these samples had been previously characterized by conventional approaches (HRM and/or direct sequencing). Table 1 and Supplementary Table S1 present the patients' characteristics and the corresponding specimen examined in the study. All samples assessed using these techniques were considered negative for the $EGFR^{T790M}$ mutation. Specifically, 76 patients (42.5%) did not harbor any mutations, and 103 patients (57.5%) displayed an $EGFR^{activating}$ mutation: $EGFR^{del19}$ and $EGFR^{L858R}$ in 56 (54.4%) and 47 (45.6%) patients, respectively.

Table 1. Patient and specimen characteristics ($N = 179$)

Characteristics	Patients with an $EGFR^{activating}$ mutation ($N = 103$) N (%)	Patients without an $EGFR^{activating}$ mutation ($N = 76$) N (%)
Sex		
Male	38 (36.9)	55 (72.4)
Female	65 (63.1)	21 (27.6)
Age		
<60	32 (31.1)	23 (30.3)
≥60	71 (68.9)	53 (69.3)
Smoking status		
Have smoked	36 (35.0)	14 (18.4)
Smoker	9 (8.7)	32 (42.1)
Nonsmoker	55 (53.4)	25 (32.9)
Unknown	3 (2.9)	5 (6.6)
Stage		
I	40 (38.8)	24 (31.6)
II	7 (6.8)	9 (11.8)
III	9 (8.7)	14 (18.4)
IV	44 (42.7)	29 (38.2)
Unknown	3 (2.9)	0 (0)
Type of specimen		
Biopsy	51 (49.5)	26 (34.2)
Surgical specimen	48 (46.6)	50 (65.8)
Other	3 (2.9)	0 (0)
Unknown	1 (1.0)	0 (0)
Tumor cell content		
<50%	30 (29.1)	13 (17.1)
≥50%	69 (67.0)	63 (82.9)
Unknown	4 (3.9)	0 (0)
$EGFR^{activating}$ Mutation status		
None	0 (0)	76 (100)
$EGFR^{del19}$	56 (54.4)	0 (0)
$EGFR^{L858R}$	47 (45.6)	0 (0)
TKI Administration		
Yes	55 (53.4)	0 (0)
No	48 (46.6)	76 (100)

Detection of the ultra-low $EGFR^{T790M}$ mutation in lung cancer tissue samples

The 179 specimens were analyzed by ddPCR. Among our cohort, 29 samples did not meet our selection criteria (≥2 $EGFR^{T790M}$-positive droplets or ≥2,000 WT-positive droplets detected, Supplementary Fig. S1) and were therefore excluded from the study (Fig. 1). Using ddPCR, we detected the presence of $EGFR^{T790M}$ subclones among 99 (66%) tissue samples of the 150 specimens initially considered as negative, based on conventional approaches. The range of VAF values obtained (from 0.0009% to 4.2%) is presented in Table 2.

We then applied a ddPCR $EGFR^{T790M}$ threshold and divided samples into two groups: patients with an $EGFR^{T790M}$ VAF < 0.1% ($N = 101$; 67.3%) or a VAF > 0.1% ($N = 49$; 32.7%). Using this threshold, we observed that $EGFR^{T790M}$ mutation presence at a level greater than 0.1% was significantly associated with the concomitant presence of an $EGFR^{activating}$ mutation in the tumor

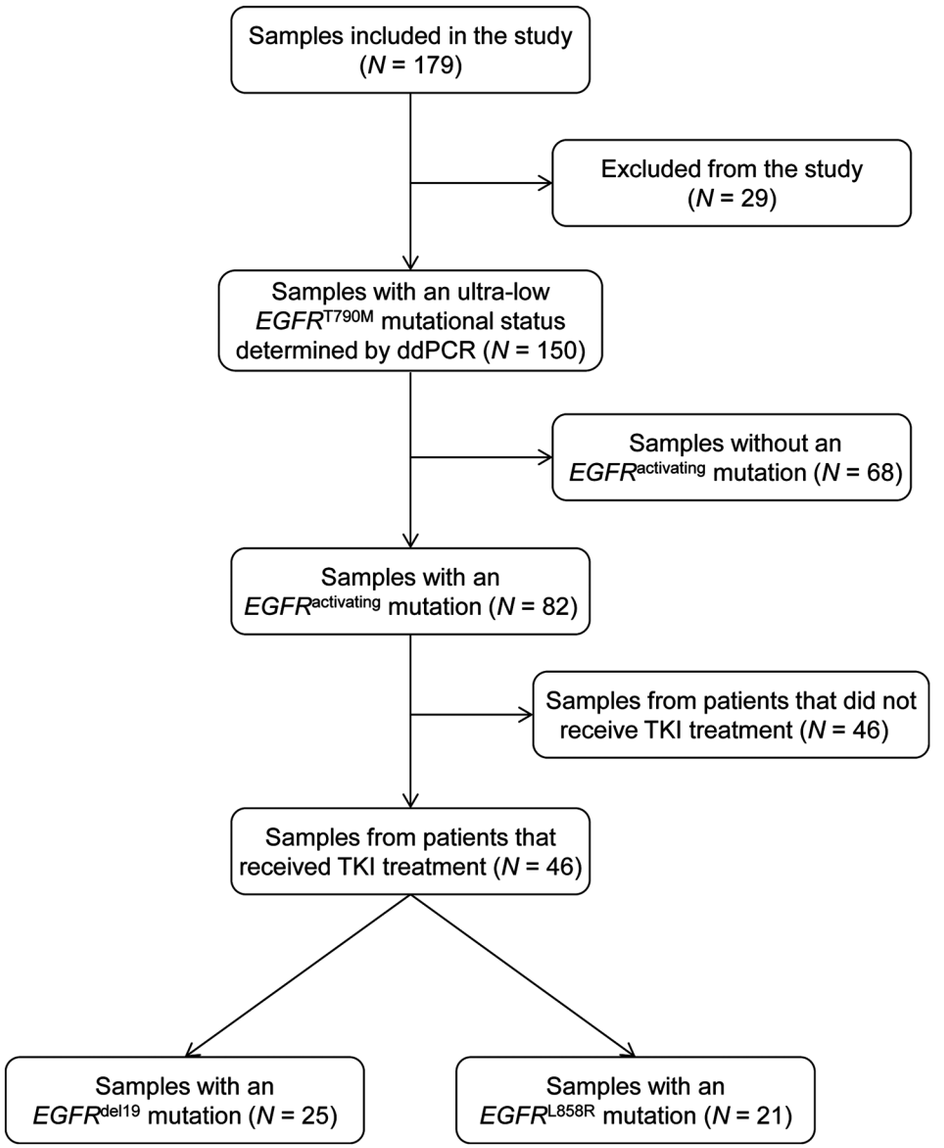

Figure 1.
Flowchart of sample selection and subgroup implementation.

sample ($P = 0.029$, χ^2 test; Table 2). This finding supports the hypothesis that administration of first-generation TKIs to these patients may have contributed to the development of acquired resistance through the selection of preexisting *EGFR*^{T790M}-resistant cellular subclones. Therefore, patients with *EGFR*^{T790M} VAF > 0.1% were considered ultra-low *EGFR*^{T790M}-positive patients in the subsequent analyses.

Table 2. VAF of the *EGFR*^{T790M} mutation detected by ddPCR in 150 pretreated NSCLC tumor samples

EGFR^{T790M} Mutation	Patients with an *EGFR*^{activating} mutation (*N* = 82) *N* (%)	Patients without an *EGFR*^{activating} mutation (*N* = 68) *N* (%)	*P*
VAF <0.1%	49 (59.8)	52 (76.5)	0.029
VAF >0.1%	33 (40.2)	16 (23.5)	

NOTE: Significance (χ^2 test) was considered at $P < 0.05$.

Characteristics and clinical outcome of patients with EGFR^{activating} mutations and the ultra-low EGFR^{T790M} mutation

Among the 150 analyzable samples, 82 exhibited an *EGFR*^{activating} mutation, whereas 68 were negative for *EGFR*^{del19} or *EGFR*^{L858R} (Fig. 1). For all samples, no alterations were detected by conventional approaches on *KRAS*, *BRAF*, and *HER2* hotspot mutations. Of note, no rare *EGFR*^{activating} mutation has been detected in any of the patient samples through HRM and Sanger. Among the 82 patients with an *EGFR*^{activating} mutation, no significant difference was observed in clinical characteristics, including sex, age, smoking status, stage, and primary mutation status, between ultra-low *EGFR*^{T790M}-positive or ultra-low *EGFR*^{T790M}-negative patients (Supplementary Table S3). Moreover, no prognostic value in terms of PFS and OS for this cohort was observed by univariate analysis (Fig. 2A). In addition, to explore whether the detection of ultra-low EGFR^{T790M} correlates with tumor clonality and the existence of other cooccurring hotspot mutations in genes known to drive EGFR TKI

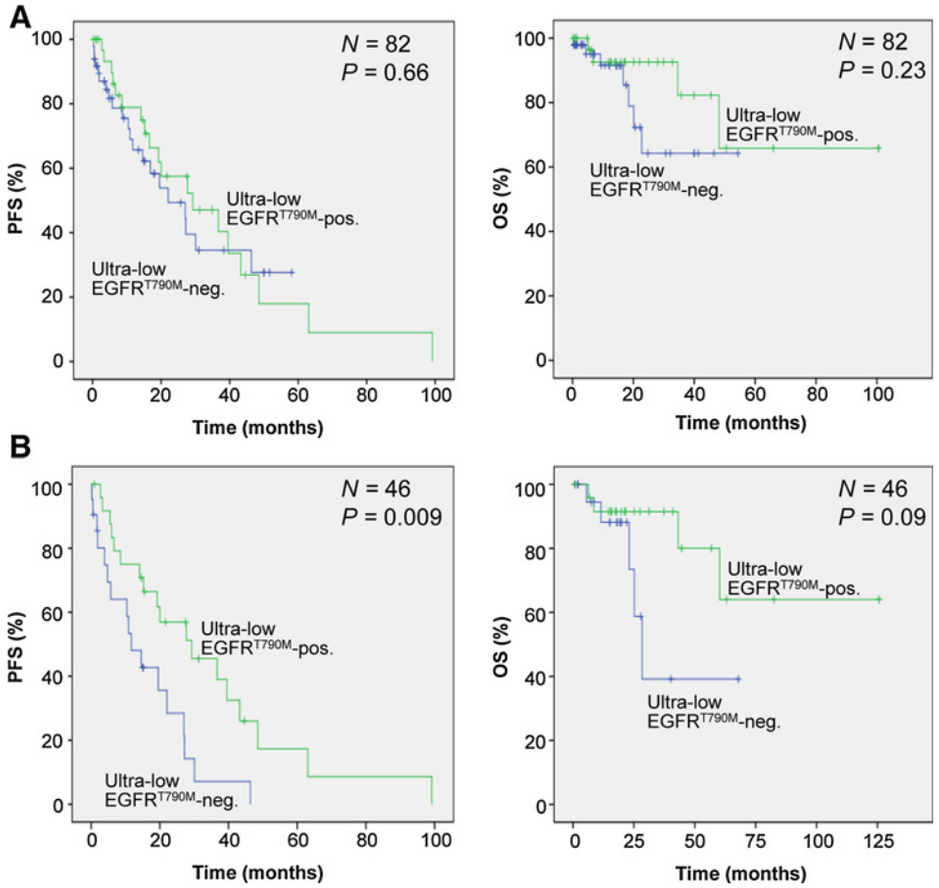

Figure 2.
Clinical outcome of patients with *EGFR*^{activating} mutations based on their pretreatment ultra-low *EGFR*^{T790M} mutation status. Kaplan–Meier analyses of patients harboring an *EGFR*^{activating} mutation (**A**) and *EGFR*^{activating}-positive patients who received EGFR TKI treatment (**B**). PFS (left) and OS (right).

resistance, we conducted an amplicon-based NGS panel targeting major oncodriver hotspots. Only 17 of the 33 samples that exhibited EGFR^{activating} and ultra-low EGFR^{T790M} had residual FFPE material available. Among them, two samples exhibited a *PIK3CA* and one sample a *SMAD4* mutation (Supplementary Tables S1 and S4).

Clinical outcome of patients harboring EGFR^{activating} mutations treated with first-generation TKIs based on ultra-low EGFR^{T790M} mutational status

Among the 82 patients who exhibited both *EGFR*^{activating} mutations, we focused on those who received first-generation TKI treatment ($N = 46$, Fig. 1). The presence of a preexisting *EGFR*^{T790M} mutation prior to any treatment was significantly associated with a longer PFS under first generation TKIs ($P = 0.009$; log-rank test; Fig. 2B and Table 3). The median of PFS of patients with an *EGFR*^{T790M}-positive mutation status was 29.2 months versus 11.7 months for *EGFR*^{T790M}-negative patients. A similar trend was also observed in terms of OS ($P = 0.09$, log-rank test; Fig. 2B). Interestingly, when the prognostic value of clinical parameters was assessed for PFS in univariate analysis, *EGFR*^{activating} mutation status appeared as another prognostic factor, with a P value of near significance ($P = 0.063$; Table 3). Both parameters (*EGFR*^{T790M} mutation and *EGFR*^{activating} mutation) were subsequently entered in a multivariate Cox model, though only the prognostic significance of the *EGFR*^{T790M} mutation status persisted in the model, suggesting that compared with *EGFR*^{activating} mutation status, determination of this molecular alteration at an ultra-low level may represent a more informative independent prognostic marker.

Given that different outcomes between *EGFR*^{del19} and *EGFR*^{L858R} mutations have been observed in patients treated with

Table 3. Univariate analysis of the prognostic value of the presence of the ultra-low *EGFR*^{T790M} mutation and clinical parameters with regard to PFS

		Univariate analysis	
	N	**HR (95% CI)**	***P***
Ultra-low level *EGFR*^{T790M} mutation (*EGFR*^{T790M} positive; *EGFR*^{T790M} negative)	46	2.522 (1.235–5.147)	0.009
Sex (male; female)	46	1.273 (0.626–2.589)	0.504
Age (<60; ≥60-year old)	46	1.038 (0.505–2.131)	0.919
Smoking history (smoker/have smoked; nonsmokers)	45	0.846 (0.412–1.737)	0.648
EGFR^{activating} Mutation (*EGFR*^{del19}; *EGFR*^{L858R})	46	2.041 (0.949–4.387)	0.063

NOTE: Significance (log-rank test) was considered at $P < 0.05$.
Abbreviation: 95% CI, 95% confidence interval.

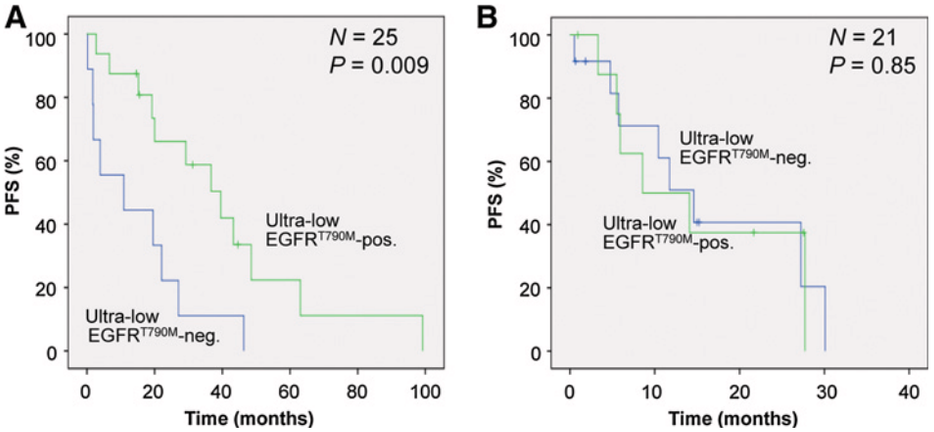

Figure 3.
Preexisting ultra-low *EGFR*^{T790M} mutation is a marker of good prognosis in patients harboring an *EGFR*^{del19} mutation under first-generation TKI treatment. Kaplan–Meier analyses of PFS in patients with ultra-low *EGFR*^{T790M} mutation status (**A**) harboring an *EGFR*^{del19} mutation or (**B**) the *EGFR*^{L858R} mutation.

first-generation TKIs (25, 26), we further assessed the prognostic value of ultra-low *EGFR*^{T790M} mutation status in *EGFR*^{del19}- and *EGFR*^{L858R}-positive patients (Fig. 1). Among patients who harbored *EGFR*^{del19}, those who carried a concomitant ultra-low *EGFR*^{T790M} mutation exhibited a significantly longer PFS (median of PFS of 39.5 months *vs.* 10.9 months, respectively; $P = 0.009$, log-rank test; Fig. 3A) and OS ($P = 0.006$, log-rank test; Supplementary Fig. S3A). However, no significant difference in PFS and OS was observed between ultra-low *EGFR*^{T790M}-positive and ultra-low *EGFR*^{T790M}-negative cases among patients with the *EGFR*^{L858R} mutation ($P = 0.86$ and $P = 0.35$, respectively, log-rank test; Fig. 3B; Supplementary Fig. S3B).

Finally, a signature combining *EGFR*^{del19}- or *EGFR*^{L858R}-positive cases with ultra-low *EGFR*^{T790M} mutational status was evaluated for PFS, with significant differences noted among the four groups ($P = 0.015$, log-rank test; Fig. 4A). According to Kaplan–Meier curves, patients were allocated into two groups: (i) those who harbored both *EGFR*^{del19} and ultra-low *EGFR*^{T790M}

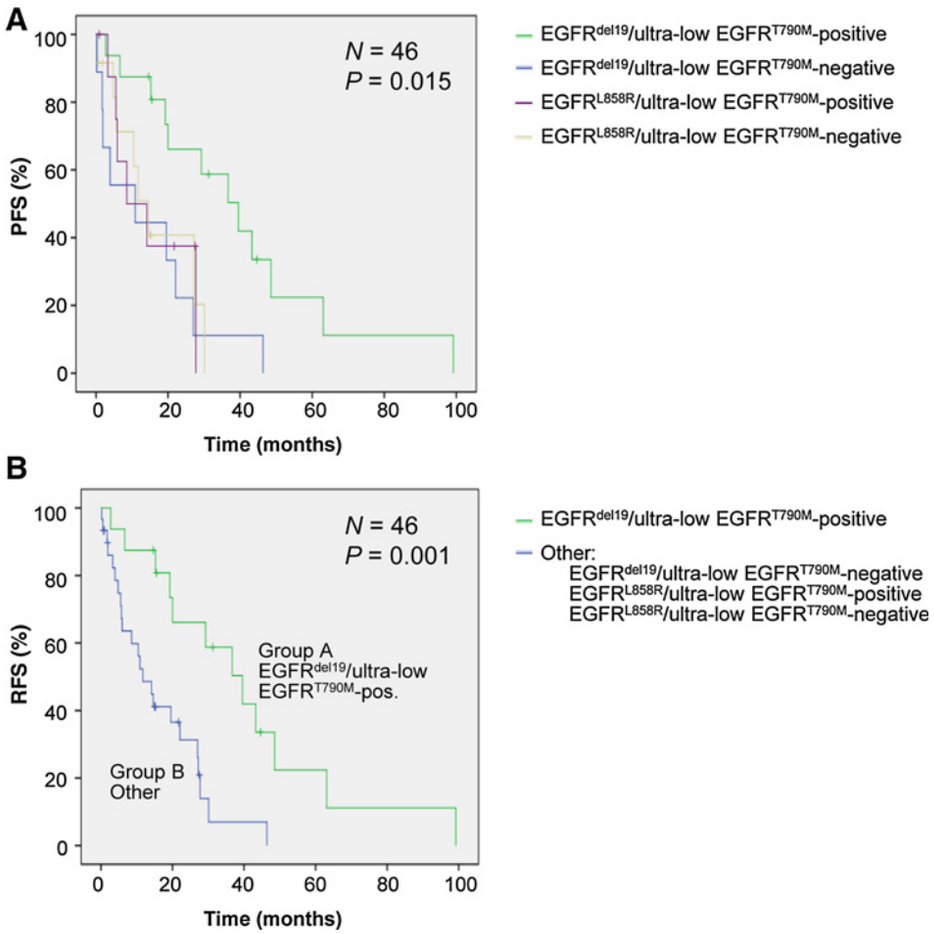

Figure 4.
Prognostic value of a signature combining the specific *EGFR*^{activating} and *EGFR*^{T790M} mutational status in patients treated with first-generation TKIs. **A,** Kaplan–Meier analyses of PFS in four groups assessed separately. **B,** The *EGFR*^{del19}/pretreatment ultra-low *EGFR*^{T790M} mutation signature is proposed as the model with the best fit in terms of PFS.

mutations (group A), and (ii) those who did not harbor these mutations (group B). Interestingly, we observed that group A patients had a better outcome in terms of PFS when compared with group B patients (median of 39.5 months vs. 11.7 months, respectively; $P = 0.001$, log-rank test; Fig. 4B). Determination of the best model was evaluated using the likelihood ratio test. The presence of both $EGFR^{del19}$ and ultra-low $EGFR^{T790M}$ mutations was identified as the best fit for predicting clinical outcomes of patients treated with first-generation TKIs (likelihood = 189.39) relative to the model with the ultra-low $EGFR^{T790M}$ mutation status alone or the activating mutation alone (likelihood = 193.52 with $P = 0.042$ and likelihood = 196.619 with $P = 0.0071$, respectively). The same results were obtained for OS.

Discussion

The $EGFR^{T790M}$ mutation is rarely found simultaneously with other sensitizing mutations, such as the $EGFR^{L858R}$ point mutation or $EGFR^{del19}$ prior TKI treatment. Moreover, the reported frequency of the $EGFR^{T790M}$ mutation varies widely in the literature: most reported frequencies are at approximately 10%, though some data indicate that this mutation can occur in greater than 70% of lung adenocarcinomas. Indeed, the frequency of $EGFR^{T790M}$ detection was 0.4% to 8% when applying direct sequencing (11, 19–21, 27–31), whereas the values increased to 31.5%, 35%, 38%, 65.3%, 68%, and 79% with more accurate methods such as MALDI-TOF MS (7, 21), the TaqMan assay (14), SARMS (13), peptide nucleic acid–clamping PCR (12), locked nucleic acid–based PCR (32), and colony hybridization (33), respectively. These variations are the result of varying sensitivities between these techniques and their ability to efficiently identify rare subpopulations within a multitude of cancer cell clones.

Using ddPCR, Watanabe and colleagues detected the $EGFR^{T790M}$ mutation in 79.9% of cases (298 of 373) in a concordant population and the same ddPCR approach (21). Two other ddPCR-based studies have reported an $EGFR^{T790M}$ mutation frequency of 40% and 100% for pretreated samples assessed (34, 35); the large range of frequency might be explained by the limited size of the tested populations, with 20 and 13 cases, respectively. In this study, we observed a clear advantage of ddPCR compared with conventional techniques (HRM and direct sequencing), as the former was capable of detecting the $EGFR^{T790M}$ mutation when present at very low frequency, even as low as 0.0009% of total DNA. Using this highly sensitive technique, we successfully identified the presence of a preexisting $EGFR^{T790M}$ mutation in 66% (99 of 150) of samples. Most of these mutations exhibited frequencies less than 0.1% (one mutant molecule in a background of 999 WT molecules), which is undetectable with the methods commonly used in clinical laboratories, thus explaining why samples were considered to be $EGFR^{T790M}$-negative according to conventional approaches in our study, as also noted in most clinical trials of EGFR TKIs. However, one of the major concerns that can be encountered when working with FFPE samples combined with highly sensitive methods of detection is the quality of DNA. Indeed, the increased sequence artifacts characteristic of FFPE DNA makes the discrimination of true low-frequency genetic variants from artifactual changes extremely challenging (36, 37). To assess the probability of deamination events that could be responsible of the detection of artefactual ultra-low $EGFR^{T790M}$ mutation, we first performed amplicon-based NGS panel of 35 actionable genes, and observed that the number of variants was compatible with a low, degraded quality of FFPE samples. Second, we tested uracil-DNA glycosylase (UDG) pretreatment strategy on ultra-low positive $EGFR^{T790M}$ samples and observed that all samples remained positive for ultra-low $EGFR^{T790M}$, demonstrating that our samples did not exhibit a high degree of FFPE-induced damage that could be responsible of artifactual molecular alterations (38).

Overall, it remains unclear whether and how $EGFR^{T790M}$ is induced by EGFR-TKI therapy. NSCLC cells harboring the $EGFR^{T790M}$ mutation may be present as a small drug-tolerant subpopulation in cells with activating mutations prior to EGFR-TKI exposure. Sharma and colleagues first demonstrated the presence of a subpopulation of "drug-tolerant" cells characterized by a quiescent phenotype in *EGFR*-mutated cells treated with TKIs (10). This particular phenotype can emerge *de novo* and be reversed following treatment arrest (10). Furthermore, these drug-tolerant cells may provide a latent reservoir from which various resistance mechanisms, including various genetic alterations, may emerge in response to treatment (8). By monitoring the appearance and development of $EGFR^{T790M}$-resistant clones, Hata and colleagues proposed a new evolutionary model of TKI resistance based on the emergence of (i) early-resistant clones from rare preexisting $EGFR^{T790M}$-positive cells and (ii) late-resistant $EGFR^{T790M}$ clones from $EGFR^{T790M}$-negative drug-tolerant cells (9). Although this hypothesis was based on a limited number of patient-derived cell lines, further investigations with additional laboratory models and validation with clinical response data are warranted. Interestingly, when we analyzed available specimens of patients treated by first-generation TKI (11 patients with tissue biopsy, and 2 patients with blood sample), we could observe that all of them exhibited both $EGFR^{activating}$ and $EGFR^{T790M}$ alterations at relapse. These results suggest that resistance could arise from the expansion of preexisting $EGFR^{T790M}$ clones under the pressure of EGFR TKI therapy. Selection of preexistent resistant subclones that a tumor can acquire stochastically is also one of the routes through which a tumor can become TKI-resistant (9). Large genomic analyses of *EGFR*-mutated lung cancer prior to initiation of TKI treatment have demonstrated a substantial intratumoral clonal heterogeneity with subclones often containing resistance-conferring alterations in genes such as *MET*, *BRAF*, *ERBB2*, *CTNNB1*, or *PIK3CA* (39, 40). Although TKI resistance often emerges through the activation of bypass signaling pathways (41, 42), we could not correlate the detection of ultra-low $EGFR^{T790M}$ with tumor clonality and the existence of other cooccurring hotspot mutations. Together, these results revive the interest in $EGFR^{T790M}$ detection to guide patient-based optimal sequential therapy with first-generation EGFR TKI.

The clinical impact of the presence of a small proportion of clones harboring the $EGFR^{T790M}$ mutation in a heterogeneous tumor before EGFR TKI treatment remains controversial. To date, there has been no reported partial response to gefitinib or erlotinib in $EGFR^{T790M}$-positive patients among studies using standard sequencing. However, using more sensitive $EGFR^{T790M}$ detection approaches, Maheswaran and colleagues reported an association with a poor outcome in 10 $EGFR^{activating}$-positive patients with concomitant $EGFR^{T790M}$ (7.7 months) compared with 16 patients with $EGFR^{activating}$ mutant tumors without the $EGFR^{T790M}$ mutation (16.5 months, $P < 0.001$; ref. 13). Su and colleagues confirmed this observation in a cohort of 56 patients

with *EGFR*^{activating} mutations; specifically, patients with a pretreatment *EGFR*^{T790M} mutation exhibited significantly shorter PFS than did patients without the *EGFR*^{T790M} mutation (6.7 vs. 10.2 months, respectively, $P = 0.03$; ref. 11). In addition, Rosell and colleagues reported PFS of 12.0 and 18.0 months in a cohort of patients with and without the *EGFR*^{T790M} mutation, respectively ($P = 0.05$; ref. 14), and Costa and colleagues found that PFS in response to erlotinib was 9.7 months for patients with the *EGFR*^{T790M} mutation and 15.8 months for those without the mutation ($P = 0.001$). These observations were sustained in two meta-analysis studies (15, 16). In contrast, a significantly longer PFS for *EGFR*^{T790M}-positive patients compared with *EGFR*^{T790M}-negative patients was observed by Oxnard and colleagues in a cohort of 93 individuals after progression on TKI ($P = 0.008$; ref. 43). Kuiper and colleagues also reported that patients developing the *EGFR*^{T790M} mutation post-TKI biopsy exhibited a longer median PFS compared with *EGFR*^{T790M}-negative patients (14.2 vs. 11.1 months respectively, $P = 0.034$) and a longer OS (45.9 months vs. 29.8 months respectively, $P = 0.213$; ref. 44). In addition, in a cohort of 73 EGFR TKI patients who underwent rebiopsy, Matsuo and colleagues reported a PFS of 13.6 versus 7.0 months in those with and without *EGFR*^{T790M}, respectively (45). Finally, Fujita and colleagues observed a significantly longer PFS in *EGFR*^{T790M}-positive patients depending on the level of the positive signal (33). By dividing the *EGFR*^{T790M} mutation signal into strongly positive, modestly positive, and negative, these authors observed that patients with a strongly positive signal had a longer PFS compared with those with a modest positive signal ($P = 0.019$) or without *EGFR*^{T790M} ($P = 0.0097$), suggesting that the abundance but not the presence of preexisting *EGFR*^{T790M} in untreated cancer cells impacts the benefits of EGFR TKI treatment. In our ddPCR study, we defined a specific threshold of 0.1% and segregated patients with longer PFS among those with *EGFR*^{activating} mutations. Therefore, we believe that the establishment of a threshold is of highest interest to future clinical trials, in particular to accurately determine the prognostic value of *EGFR*^{T790M} mutation presence.

Here, we observed the *EGFR*^{T790M} mutation to be associated with higher PFS and OS in patients harboring *EGFR*^{del19} compared with those harboring *EGFR*^{L858R}. One attractive explanation would be that a small reservoir of *EGFR*^{T790M} cells preexisting in a complex mixture of heterogeneous tumoral clones may mediate different specific cellular features (9). If it occurs *in trans*, this could explain why tumors that harbor both an *EGFR*^{activating} mutation and *EGFR*^{T790M} mutation are not resistant to EGFR TKIs. One could then imagine that a subclone harboring concurrent *EGFR*^{activating} and *EGFR*^{T790M} mutations could influence the growth of other clones through either paracrine interactions (chemokines, cytokines, exosomes) or inducing changes in tumor microenvironment. These complex interactions between several clones could be subtly modulated by other mechanisms, such as noncoding RNAs' expression or epigenetic alterations, that could therefore have an important impact on, for example, the response to treatment, and the kinetic of relapse. In addition, when present in *cis* to the *EGFR*^{activating} mutation, the *EGFR*^{T790M} mutation confers gefitinib resistance and activates the downstream PI3K/Akt signaling pathway (46). One would suggest to better investigate the effect of the *EGFR*^{T790M} position in regards of the *EGFR*^{activating} mutation in patient samples. Further investigations using follow-up clinical data is needed to understand the clinical significance of these early and low-level mutations. To our knowledge, the impact of concomitant *EGFR*^{del19} and ultra-low *EGFR*^{T790M} on PFS and OS has not been assessed before. The observation that *EGFR*^{del19} patients have a better outcome than do *EGFR*^{L858R}-positive patients under first-generation TKI therapy could be explained by the presence of an ultra-low *EGFR*^{T790M} subgroup.

Recently, third-generation EGFR TKIs, including osimertinib, rociletinib, and ASP8273, have been proposed as promising therapies with antitumor activity for both *EGFR*^{activating} and *EGFR*^{T790M} mutations. In a phase II clinical trial including 199 *EGFR*^{T790M}-positive patients, Goss and colleagues reported an objective response to osimertinib treatment in 140 patients (70.3%) (47). The Aura3 phase III trial involved 419 patients with *EGFR*^{T790M}-positive lung cancer who exhibited disease progression after first-line EGFR TKI therapy and demonstrated that the median duration of PFS was significantly longer with osimertinib compared with platinum chemotherapy plus pemetrexed (48). Thus, third-generation EGFR TKIs as a first-line treatment may have the potential to eradicate tumors harboring both *EGFR*^{activating} and pretreatment *EGFR*^{T790M} mutations, rendering first-generation TKIs insufficient or not suitable for *EGFR*^{activating} patients. This finding was recently demonstrated in the FLAURA trial, whereby osimertinib exhibited greater efficacy compared with standard EGFR TKIs in the first-line treatment of *EGFR*^{activating}-positive advanced NSCLC (18). The PFS curves were clearly separated from the beginning of the trial, suggesting that osimertinib can both target preexisting and acquired *EGFR*^{T790M} clones. Nevertheless, nowadays first- and second-generation TKIs are still routinely used in many countries as no improvement in OS has been demonstrated yet in the FLAURA trial. Moreover, as it is the case with first- and second-generation TKIs, resistance to osimertinib eventually emerges through gain of additional alterations in cancer cells. There is a clear need to understand the molecular mechanisms underlying acquired resistance to osimertinib to prolong survival in patients with EGFR-mutated lung cancer. Knowledge on the frequency of ultra-low EGFR^{C797S} before TKI introduction as well as the influence of the early acquisition of EGFR^{C797S} pretreatment on PFS should be further investigated in future studies.

The underlining biological mechanisms are likely highly complex. In early- and late-emerging *EGFR*^{T790M} clones, differential levels of apoptosis were observed depending on the generation of TKI administered (9): early-emerging *EGFR*^{T790M} clones were sensitive to third-generation TKIs but resistant to first-generation TKIs, whereas late-emerging *EGFR*^{T790M} clones were less responsive to third-generation EGFR inhibitors (9). Overall, an ultrasensitive and quantitative assay, such as ddPCR, would be advantageous for precisely detecting patients harboring ultra-low *EGFR*^{T790M} mutations who may benefit from third-generation EGFR TKIs, eliminating early-emerging *EGFR*^{T790M} reservoir cells and delaying the evolution of acquired resistance. In contrast, ultra-low *EGFR*^{T790M}-negative patients could be preferentially treated with first-generation TKIs as first-line treatment.

A clinical trial is currently ongoing (ARCHER 1050) to evaluate the efficiency and safety of the second-generation dacomitinib versus gefitinib as first-line therapy in patients with newly diagnosed, locally advanced, or metastatic NSCLC with *EGFR*^{activating} mutations. First reports have showed promising results as a first-line treatment (49). Finally, a first- or second-generation EGFR TKIs followed by osimertinib should become a standard

treatment strategy for chemotherapy-naïve patients with *EGFR*^{mutated} NSCLC; however, the best sequence for administration of these drugs with regard to maximization of the duration of the EGFR signaling inhibition has not yet been determined. In addition, a support to help the selection of patients at diagnosis, who are likely to develop *EGFR*^{T790M} after treatment with a first- or second-generation EGFR TKIs, needs to be proposed.

In conclusion, the data presented in this study suggest the potential of ddPCR as a high-resolution diagnostic tool for patient selection with the aim of enabling more personalized therapy. Using a highly sensitive ddPCR method, we identified a large majority of patients with NSCLC harboring concomitant *EGFR*^{activating} and *EGFR*^{T790M} mutations before EGFR TKI introduction. Interestingly, we demonstrated that detection of ultra-low *EGFR*^{T790M} in TKI-naïve patients can define a subset of patients with a distinct clinical outcome. Finally, we observed that the presence of both ultra-low *EGFR*^{T790M} and *EGFR*^{del19} mutations was significantly associated with a strong response rate to first-generation TKIs and a longer PFS. Altogether, given that different therapeutic strategies are required to overcome *EGFR*^{T790M}-mediated acquired resistance, we believe that our findings will have relevant clinical implications for patient management and that ultra-low *EGFR*^{T790M} mutation detection should be taken into account in the design and interpretation of future clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.A. Vendrell, J. Mazieres, R. Senal, J.-L. Pujol, B. Roch, J. Solassol
Writing, review, and/or revision of the manuscript: J.A. Vendrell, J. Mazieres, X. Quantin, J.-L. Pujol, S. Godreuil, E. Coyaud, J. Solassol
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