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ORIGINAL ARTICLE

Independent prognostic value of ultra-sensitive quantification of tumor pre-treatment T790M subclones in *EGFR* mutated non-small cell lung cancer (NSCLC) treated by first/second generation TKI, depends on variant allele frequency (VAF): results of the French Cooperative Thoracic Intergroup (IFCT) Biomarkers France project

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Abstract

Objectives: T790M mutations in *EGFR*-mutated non-small cell lung cancer (NSCLC) account for nearly 50% of acquired resistance mechanisms to EGFR-TKIs. Earlier studies suggested that tumor T790M could also be detected in TKI-naïve *EGFR*-mutated NSCLC. The aim of the study is to assess the prevalence and clinical significance of quantification of tumor pre-treatment T790M subclones.

Materials and methods: We analyzed 366 *EGFR*-mutated NSCLC patients of the real-life IFCT Biomarkers France study with available pre-treatment formalin-fixed paraffin-embedded (FFPE) tumor DNA before treatment by first/second-generation EGFR-TKI. We used ultra-sensitive Droplet Digital Polymerase Chain Reaction (ddPCR) QX200 (BIO-RAD®, Hercules, CA, USA). All samples were tested in duplicate.

Results: ddPCR identified T790M in 19/240 specimens (8%). T790M-positive and T790M-negative populations were not different for clinical baseline characteristics. T790M Variant Allele Frequency (VAF) was $\geq 0.01\% < 0.1\%$, $\geq 0.1\% < 1\%$, $\geq 1\% < 10\%$, and $\geq 10\%$ in five (26.3%), six (31.6%), six (31.6%), and two (10.5%) patients, respectively. T790M VAF was $> 0.1\%$ in 11/13 (84%) patients with rapid (< 3 months) or usual progression (3-20 months) compared to 0/3 with low progression (> 20 months) ($p=0.02$). In a Cox model, T790M mutation positivity was correlated with overall survival (OS) and progression-free survival (PFS) for $10\% > \text{VAF} \geq 1\%$ (hazard ratio [HR]=2.83, 95% confidence interval [CI] 1.13-7.07, $p=0.03$; HR=3.62, 95%CI 1.43-4.92, $p=0.007$, respectively) and for $\text{VAF} \geq 10\%$ (HR=19.14, 95%CI 4.35-84.26, $p<0.001$; HR=17.89, 95%CI 2.21-144.86, $p=0.007$, respectively).

Conclusion: Ultra-sensitive detection of tumor T790M mutation concerned 8% of *EGFR*-mutated TKI-naïve NSCLC patients and has a negative prognostic value only for T790M VAF over 1%.

Key words: Non-small cell lung cancer (NSCLC), EGFR mutation, T790M, variant allele frequency (VAF), droplet digital PCR (ddPCR)

1. Introduction

Advanced non-small cell lung cancer (NSCLC) patients whose tumor harboring Epidermal Growth Factor Receptor (*EGFR*)-activating mutations in exon 19 (Del19) and exon 21 (L858R) are characterized by a 70% overall response rate and prolonged progression-free survival (PFS) to first/second-generation *EGFR*-TKI (Tyrosine Kinase Inhibitor) treatment [1–5]. Yet, patients develop acquired resistance due to *EGFR* T790M mutation (c.2369 C>T at exon 20), occurring in over 50% [6]. Third-generation *EGFR*-TKI osimertinib is now considered to be the standard of care for such T790M-positive NSCLC patients who have progressed upon first-line *EGFR*-TKI therapy [7]. More recently, osimertinib proved more effective than standard first/second-generation *EGFR*-TKIs in first-line treatment of *EGFR* mutation-positive advanced NSCLC [8].

The natural history of *EGFR* T790M mutation appears highly complex [9]. While T790M mutation represents a good post-first/second generation *EGFR*-TKI prognostic factor, there have been contradictory results regarding the predictive/prognostic value of the identification of pre-treatment T790M mutated clones [10-13]. Acquired resistance under first/second-generation *EGFR*-TKI therapy caused by the *EGFR* T790M gatekeeper mutation can occur either due to selection of pre-existing T790M-positive clones or a genetic evolution of initially T790M-negative drug-tolerant cells [9]. Nevertheless, T790M detection was not consistent in sequential re-biopsies following progression in such situations [14]. The emergence of T790M wild-type clones under second-line third-generation *EGFR*-TKIs is reported in up to half of the patients with T790M, hence named T790M wild-type progression and suggests tumor heterogeneity [15, 16]. The intra-tumor heterogeneity of T790M mutation could also explain the presence of such pre-treatment mutation in *EGFR*-TKI treatment-naïve NSCLC patients.

Pre-treatment tumor T790M mutations were reported to occur with varying prevalence, ranging from <1% using conventional Sanger DNA sequencing to 80% using more sensitive allele-specific techniques [17–20]. The detection of the T790M mutation in FFPE sample at a low allelic frequency is challenging due to the fact that it is a C>T transition and mimics a FFPE artefact. Data on pre-treatment T790M mutation frequency proved discordant, even when using the same molecular techniques [21]. Droplet Digital Polymerase Chain Reaction (ddPCR) is a highly quantitative ultrasensitive gene mutation detection method with a theoretical sensitivity of 0.005% [22]. The ddPCR has been validated for detecting T790M mutations in circulating cell-free DNA after progression upon first-line EGFR-TKI [23].

This study based on a one-year large nationwide real-life French Cooperative Thoracic Intergroup (IFCT) screening program in 2012 [4, 24], sought to quantify pre-treatment tumor T790M mutation using ultra-sensitive ddPCR and to investigate its impact on clinical outcomes of *EGFR*-mutated NSCLC patients treated by first/second-generation EGFR-TKI.

2. Materials and methods

2.1. Study population

This study is an ancillary project of the prospective IFCT Biomarkers France program. We selected 336 *EGFR*-mutated advanced NSCLC patients from all the IFCT Biomarkers France cohort with available tumor DNA after locally molecular analysis and treated by first/second-generation EGFR-TKI [4, 24]. In order to separate patients with rapid progression (PFS \leq 3 months) from patients with late progression (PFS \geq 20 months), we classified patients in three groups depending of EGFR-TKI PFS [1-5]. This study was approved by the national

committee for the protection of persons (CPP), French Advisory Committee on Information Processing in Material Research in the Field of Health (CCTIRS), and National Commission of Informatics and Liberty (CNIL).

2.2. Detection of T790M mutation by ddPCR

All reactions were prepared using the ddPCR Supermix for Probes (BioRad, Hercules, CA, USA) and performed in duplicate, by one centralized centre (Supplementary Data). Two different labeled probes are tested in a single reaction (BioRad®), one to detect the mutation (6-carboxy-fluorescein, FAM label-blue) and the other to detect the wild-type allele (Hexachloro-fluorescein, HEX label-green). Quantitative value of T790M was determined by the variant allele frequency (VAF). VAF of T790M mutation was calculated based on the ratio between the T790M (FAM+/HEX-) droplet number and total full droplet number, with a theoretical detection limit of 0.005% per well (1/20.000 droplets generated in each well). For validation, DNA extracted from formalin-fixed paraffin-embedded (FFPE) colon cancer tumor samples (n=30) were tested with a final threshold VAF value for positive T790M mutation at 0.03% (Supplementary Data). False-positives (FAM+/HEX+) or discordant replicates (defined as one positive well with positive droplets and one non informative well with any positive droplet) were excluded.

2.3. Statistical analysis

Results were expressed as medians for continuous variables and percentages for categorical variables, with comparisons made using chi-squared or Fisher's exact tests for categorical variables, and Student's t-test or ANOVA for continuous variables, with a significance level at $p < 0.05$. Survival curves were estimated using the Kaplan–Meier method. Overall survival

(OS) and progression-free survival (PFS) were previously defined [24]. Disease control rate (DCR) was defined as the percentage of patients with stable disease, partial response, or complete response, and overall response rate (ORR) as that of patients with partial and complete response. A Cox model was applied to estimate hazard ratios (HR) and 95% confidence intervals (CI). SAS software, Version 9.4 (SAS Institute, Cary, NC), was employed.

3. Results

3.1. Patient characteristics, tumor pre-treatment EGFR T790M mutation detected by ddPCR, and survival

Our study is a prospective multi-centre national prospective study of 336 *EGFR*-mutated NSCLC patients from the Biomarkers France project, all treated by EGFR-TKI. We re-tested all available DNA which collected results of real-life NSCLC tumors testing during one year, in 2012 (Figure S2, flow-chart, Supplementary Data). Common *EGFR* mutations are represented by EGFR del19 (exon 19) and L858R (exon 21) mutations. Patients with *EGFR* exon 20 mutations other than T790M in their tumor were excluded from response and survival analyses. Among the 240 DNA from NSCLC patients, 96 DNA were not included with 71 patients excluded for insufficient number of droplets, probably due to a very low available DNA quantity after local analysis. Samples were analyzed only if a minimal total droplet number ≥ 100 /well was reached. Overall, 240 NSCLC patients with *EGFR* mutated tumors were selected for ddPCR analysis of FFPE lung tumor DNA, who did not differ from the whole *EGFR* Biomarkers France cohort, except disease stage and performance status (Table S1, Supplementary Data). We identified 19 patients harboring a pre-treatment T790M

mutation in their tumour (8%). After including 21 cases with discordant replicates could be due to Poisson's law, our incidence increased to 15% (39/261) (data not shown). Clinical and biological characteristics were similar between patients with pre-treatment T790M-positive and T790M-negative tumors (Table 1). T790M-positive tumors were reported in 1/7 *EGFR* exon 18 (14.2%), 9/121 *EGFR* exon 19 (7.4%), 1/7 *EGFR* exon 20 (14.2%), and 8/105 *EGFR* exon 21 (7.6%) of *EGFR*-mutated tumors. The median PFS with first/second-generation EGFR-TKI was significantly shorter in patients with pre-treatment T790M-positive tumors compared to patients with T790M-negative tumors (8.5 months, 95% CI: 2.9-18.4 versus 13.1 months, 95% CI: 10.8-15.4) ($p=0.045$) (Figure 1A). The median OS was significantly shorter in patients with pre-treatment T790M-positive tumors compared to patients with T790M-negative tumors (11.6 months, 95% CI: 7.8-29.8 versus 24.8 months, 95% CI: 19.6-29.1) ($p=0.005$) (Figure 1B). On multivariate analysis, PFS with first/second-generation EGFR-TKI demonstrated death risk to be significantly higher in patients with pre-treatment T790M-positive tumors (HR=2.08; 95% CI: 1.17-3.72, $p=0.01$) (Table S2A, Supplementary Data), which was also the case for OS (HR=2.42; 95% CI: 1.371-4.26, $p=0.002$) (Table S2B, Supplementary Data). Tumor pre-treatment T790M mutation was a poor-response factor in terms of disease control rate (DCR) with first and second-line EGFR-TKIs ($p=0.045$ and $p=0.03$, respectively) (Table S3).

3.2. Quantification of tumor pre-treatment EGFR T790M mutation by fractional abundance (FA) percentage

Using ddPCR as a highly sensitive quantitative technique, we reported the results of the 19 pre-treatment T790M-positive tumors with T790M VAF (Figure 2A). The median VAF was 0.37% and mean FA 5.69%, with a minimal VAF of 0.03% and maximal VAF of 51.33%.

The 19 T790M-positive cases were categorized depending on VAF: VAF $\geq 0.01\% < 0.1\%$, $\geq 0.1\% < 1\%$, $\geq 1\% < 10\%$, $\geq 10\%$ in five (26.3%), six (31.6%), six (31.6%), and two (10.5%) patients, respectively, with no difference in T790M VAF among the *EGFR*-mutated exons (Table S4, Supplementary Data).

3.3. Variant Allele Frequency (VAF) effect of tumor pre-treatment T790M mutation on first/second generation EGFR-TKI treatment response and PFS duration in patients with NSCLC with EGFR-activating mutations

The mean T790M VAF differed in cases of progressive disease (17%) and partial response under EGFR-TKI (0.87%) ($p=0.06$) (Figure 2B). T790M VAF significantly differed in terms of disease control rate (DCR) under first- and second-line EGFR-TKI ($p=0.02$ and $p=0.03$, respectively) (Table S5). Pre-treatment T790M mutation tended to be more frequent in patients with rapid (under 3 months) ($n=6/31$, 19.4%) versus those with usual (3-20 months) and slow progression (over 20 months) ($n = 7/111$, 6.3%, and $n=3/39$, 7.7% respectively) (Table 2). Mean T790M VAF was significantly lower in patients with slow progression (0.05%) compared to usual (2.57%) or rapid progression (6.36%) ($p=0.05$) (Figure 2C). Patients with slow progression exhibited T790M VAF under 0.1% and those with usual or rapid progression exhibited T790M VAF $\geq 0.1\%$ (6/7 [85.7%] and 5/6 [83.3%] cases, respectively) ($p=0.028$) (Table 2).

3.4. Variant Allele Frequency (VAF) effect of tumor pre-treatment EGFR T790M mutation on survival in NSCLC patients with EGFR activating mutations

Median PFS under first/second generation EGFR-TKI significantly differed depending on T790M VAF, with a lower PFS only in cases with VAF >1% cases ($p < 0.001$) (Table S5, Supplementary Data). Upon multivariate analysis, PFS under first/second generation EGFR-TKI demonstrated death risk to be significantly higher only in pre-treatment T790M-positive tumors with a VAF between 1%- 10% (HR 3.62; 95% CI, 1.43-4.92, $p=0.007$) and a VAF >10% (HR 17.89; 95% CI, 2.21-144.88, $p=0.007$) (Table 3A). Median OS also significantly depended on T790M VAF, with a lower OS in VAF >1% cases ($p < 0.001$) (Table S5, Supplementary Data). Upon multivariate analysis, OS demonstrated death risk to be significantly higher only in pre-treatment T790M-positive tumors with a VAF% between 1-10% (HR 2.83; 95% CI, 1.13-7.07, $p=0.03$) and a VAF% >10% (HR 19.14; 95% CI, 4.35-84.26, $p < 0.0001$) (Table 3B).

4. Discussion

This prospective study investigates for the first time the clinical relevance of ultra-sensitive quantification of pre-treatment tumor *EGFR* T790M mutated subclones in real-life advanced Caucasian and Asian NSCLC patients all treated by first/second generation EGFR-TKIs. Using ultra-sensitive ddPCR, we identified tumor pre-treatment T790M mutation in 8% (19/240) of cases with no statistical difference in clinical characteristics found between patients with or without pre-treatment tumor T790M mutations. Ultra-sensitive quantification of tumor pre-treatment T790M mutations was an independent prognostic factor of OS and PFS, yet only for T790M with VAF of 1-10% and VAF >10%. Actually, with the availability of third generation EGFR-TKI, the best EGFR-TKI sequence algorithms in *EGFR* mutated NSCLC in real life patients still need to be defined, i.e. taking into account the quantification of pre-TKI T790M-mutated subclones tumor composition.

4.1. Frequency of T790M pre-treatment mutation

Different detection methods reported variable baseline *EGFR* T790M mutation frequencies. This variability could first be accounted for by the assays' differing sensitivity and their ability to identify minor within-tumor mutated clones [21, 25]. Using direct sequencing, several studies reported tumor baseline T790M mutation incidence to be 0.4-3% [19, 26, 27] and using other methods with greater sensitivity to be from 4-38% to 65% [25; 28]. A 79% frequency was obtained using colony hybridization with 0.01% sensitivity, but due to this level of tumor basal T790M mutations' lack of meaning, the cut-off sensitivity was raised by the authors to 0.5%, resulting in 22.9% tumor baseline T790M mutation [12, 29]. This result highlights the importance of the cut-off value of molecular techniques for the detection of T790M mutation. Secondly, studies using the same molecular technique may obtain different

pre-treatment T790M incidences. Using MALDI-TOF technology, T790M pretreatment mutation was ranging from 2-31% [19, 25] and using ddPCR from 66 to 79.9% [30, 31]. The data could differ due to cohort differences regarding patient number, stage, ethnicity, or treatments. The number of tested patients was mostly <100, with most studies involving Asian NSCLC cohorts [25, 26, 30]. In our study, ddPCR revealed that 8% (19/240) patients had tumor preexisting T790M-mutations, indicating lower pre-treatment T790M incidence compared to the two other retrospective studies using the same technology in different populations (373 surgically treated NSCLC for one and 179 all stages - with only 46 patients treated by EGFR TKI - for the other one) [30, 31]. Finally, our incidence of pre-treatment T790M mutation seems to be less prevalent than expected as more than 50% of acquired resistance is related to major tumor T790M-mutated clones.

4.2. Quantification of T790M pre-treatment mutation

Intra-tumoral heterogeneity at a molecular level could also explain these different incidences and could be approached by quantitative technology. One study used a MALDI-TOF technology (31/124, 25% positive T790M pre-treatment cases) with a quantitative approach by dividing T790M positive mutant tumors into two populations, high (n=9) versus low (n=22) according to a cell-line mixture study [20]. In our study using ultra-sensitive ddPCR, level of T790M mutation was quantified and categorized by VAF as usually done [30]. We observed an equal repartition of pre-treatment T790M FA in different categories: 0.01-0.1%, 0.1%-1%, 1-10%. One recent study using ddPCR reports 60 to 76.5% of patients with T790M VAF under 0.1% but without precision of VAF values [31]. Our results differ also from those of another quantitative study using ddPCR reporting a majority of cases with 0.01% pre-treatment T790M FA [30]. This work did not study response rate or survival.

4.3. Overall survival

Concerning OS, while longer post-progression survival after EGFR-TKI resistance was mostly observed in T790M mutation identified upon progression compared to other resistance mechanisms, the prognostic significance of tumor baseline *EGFR* T790M has been less often reported [12, 13, 31, 32]. We found tumor pre-treatment T790M to be a predictor of poor prognosis for OS, as other studies [19, 20, 25], with no reasonable explanation as yet. In one semi-quantitative study, only high tumor baseline T790M frequency was reported to be associated with worse clinical outcomes for OS [20]. In our study using ddPCR with ultra-sensitive quantitative analysis, only tumor baseline T790M until VAF at 1% had also a poor prognostic value for OS. Though clonal selection under EGFR-TKI possibly explains the increasing frequency of tumor T790M mutation from pre- to post-treatment, pre-treatment T790M mutation may prove to be heterogeneous [9]. The shorter OS of patients harboring tumor pre-treatment T790M mutations could be attributed to their shorter PFS under first/second-generation EGFR TKI.

4.4. Progression-free survival

Concerning PFS, if direct sequencing detects tumor T790M at baseline, it is now well established that erlotinib seems to not produce clinical benefits in terms of PFS or response (ORR) [25]. When using most sensitive molecular technologies, tumor pre-treatment T790M mutation were reported to negatively impact PFS in a recent meta-analysis [12], but no predictive value in terms of response rate or PFS using ARMS technology [18, 29]. The two studies reporting a positively impact on the PFS were also those reporting the highest T790M mutation incidence [12, 31]. This calls into question the value of minor baseline T790M

mutation or false-positive results [32]. In our study using ddPCR with ultra-sensitive quantitative analysis, only tumor baseline T790M until VAF at 1% had a poor prognostic value for PFS and DCR. No patient with PFS >20 months was identified as having a tumor baseline T790M mutation VAF >0.1%. Including discordant cases which correspond to no available confirmed positive cases due to lack of DNA, mean T790M mutation VAF also significantly increased from patients with PFS >20 months to patients with PFS between 3-20 months, and to patients with PFS <3 months (Figure S3, Supplementary Data). Nevertheless, Cox analysis shows no impact of T790M pre-treatment positive case, probably due to a high proportion of VAF with less than 0.1% mutated clones (data not shown). In a recent study using ddPCR, a good prognostic value (PFS and OS) was found for a signature combining activating del19 EGFR mutation and ultra-low T790M mutations [31]. As detailed T790M VAF is not available in this study, we can hypothesize that the good prognostic value could be explained by very very low VAF values. In our study, we didn't study prognostic value among the different types of *EGFR* mutated exons due to the little number of positive T790M cases. Nevertheless, we observed that *EGFR* exon 19 mutated cases presented lower pre-treatment T790M VAF values compared to *EGFR* exon 21 (Table S4). The prognostic value of pre-treatment tumor T790M mutation could perhaps differently impact prognosis depending on the incidence of different *EGFR* T790M-mutated subclone levels on response.

4.5. Predictive value

In our study, the response to EGFR-TKI were similar in patients with tumor pre-treatment T790M VAF <1% than those reported in patients with only sensitizing EGFR-mutant tumors. Conversely, mean T790M VAF significantly increased at 0.87% in partial responders to 17% in progressive disease cases. In *in vitro* experiments, different low percentages of T790M

resistant cells within the population (1% and 10%) were shown to display similar sensitivity to erlotinib as parental cells (0%) [33]. In contrast, sensitivity to erlotinib was reduced when T790M mutated clones made up >25% of the population, explaining why tumor patients harboring very low T790M mutation levels can experience an objective radiographic response to EGFR-TKI, yet nevertheless relapse.

5. Conclusions

In our series of an ancillary project of the prospective the IFCT Biomarkers France program, pre-treatment *EGFR* T790M-mutated subclones was relatively frequent (8% to 15%), yet below the 50% frequency usually reported for acquired resistance to first/second-generation EGFR-TKI [9]. These results must be validated by an external validation study. Our results imply that replacing the current binary assessment of T790M status (present versus absent) in tumor samples by an easy quantification of T790M activation mutation allele frequency by ddPCR may allow for a prognostic stratification in the perspective of third generation EGFR-TKI osimertinib [34]. As osimertinib is becoming the standard for *EGFR* mutation-positive advanced NSCLC [8], it could be interesting to detect low tumor T790M mutation at baseline, in order to anticipate specific resistance mechanisms in positive population who can lose this T790M mutation in time. In another hand, ddPCR could become an essential diagnostic tool in the future for choosing personalized EGFR –TKI sequence, i.e. pre-TKI tumor T790M VAF rate under 1% with first/second generation TKI in first line followed by osimertinib, versus pre-TKI tumor T790M FA >1% with osimertinib as first line. Quantitative detection of tumor baseline T790M mutation proved useful to more accurately assess its prognostic and predictive values, taking into account tumor cellular/molecular heterogeneity.

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Figure legends

Figure 1. Kaplan-Meier-survival curves. Curves of NSCLC patients with *EGFR* mutation divided into two subgroups according to presence or absence of ultra-sensitive detection of pre-treatment tumor T790M mutation: 1A) Progression-free survival (PFS) under first/second generation EGFR-TKI; 1B) Overall survival (OS).

Figure 2. Repartition of variant allele frequency (VAF) of pre-treatment tumor *EGFR* T790M mutation detected by droplet digital dPCR (ddPCR) in NSCLC patients harboring *EGFR* activating mutations (n= 240): 2A) For all the patients; 2B) Depending on the response to first-line first/second-generation EGFR-TKI; 2C) Depending on the progression-free survival (PFS) to first/second-generation EGFR-TKI. Mean is indicated by a cross and median by a line.

Table 1. Clinical and biological characteristics of the populations with tumor ultra-sensitive detection of pre-treatment *EGFR* T790M mutation.

Descriptive statistics			<i>EGFR</i> T790M negative (n=221)	<i>EGFR</i> T790M positive (n=19)	ALL (n=240)	p-value
Gender	Male	n (%)	68 (30.8)	6 (31.6)	74 (30.8)	0.94
	Female	n (%)	153 (69.2)	13 (68.4)	166 (69.2)	
Age (years)		n	221	19	240	0.63
		Mean ± SD	68.45 ± 10.83	68.74 ± 13.95	68.47 ± 11.08	
		Median	69.54	75.54	69.62	
		Range	[30.6-94.0]	[40.7-88.8]	[30.6-94.0]	
Asian origin						0.59
	Yes	n (%)	9 (4.6)	1 (5.6)	10 (4.2)	
	No	n (%)	186 (95.4)	17 (94.4)	203 (84.6)	
	MISSING	n	26	1	27	
Smoking						0.33
	Smoker	n (%)	28 (12.9)	2 (11.8)	30 (12.5)	
	Former smoker	n (%)	62 (28.6)	2 (11.8)	64 (26.7)	
	Non-smoker	n (%)	127 (58.5)	13 (76.5)	140 (58.3)	
	MISSING	n	4	2	6	
PS						0.50
	0	n (%)	63 (30.4)	4 (21.1)	67 (27.9)	
	1	n (%)	107 (51.7)	14 (73.7)	121 (50.4)	
	2	n (%)	31 (15.0)	1 (5.3)	32 (13.3)	
	3	n (%)	4 (1.9)	0	4 (1.7)	
	4	n (%)	2 (1.0)	0	2 (0.8)	
	MISSING	n	14	0	14	
Personal history of cancer						1.00
	Yes	n (%)	41 (18.8)	3 (15.8)	44 (18.3)	
	No	n (%)	177 (81.2)	16 (84.2)	193 (80.4)	
	MISSING	n	3	0	3	
TNM, Stage	Relapse	n (%)	28 (12.7)	4 (21.1)	32 (13.3)	0.29
	Stage IV	n (%)	193 (87.3)	15 (78.9)	208 (86.7)	
Histology	Squamous	n (%)	1 (0.5)	0	1 (0.4)	0.48
	Adenocarcinoma	n (%)	191 (86.4)	15 (78.9)	206 (85.8)	
	Large Cell	n (%)	3 (1.4)	0	3 (1.3)	
	Other	n (%)	26 (11.8)	4 (21.1)	30 (12.5)	
<i>EGFR</i> mutations	Common	n (%)	179 (81.0)	15 (78.9)	194 (80.8)	0.77
	Other	n (%)	42 (19.0)	4 (21.1)	46 (19.2)	

PS, performance status; *EGFR*, epidermal growth factor receptor.

Table 2. Correlation with tumor ultrasensitive detection of pre-treatment *EGFR* T790M mutation and progression-free survival duration with first/second generation TKIs (*n*=181), depending on T790M quantification.

T790M negative versus positive				
T790M status	PFS \geq 20 months	PFS 3-20 months	PFS \leq 3 months	<i>P</i> -value
T790M negative	36 (92.3%)	104 (93.7%)	25 (80.6%)	0.09
T790M positive	3 (7.7%)	7 (6.3%)	6 (19.4%)	
T790M variant allele frequency (VAF)				
T790M VAF	PFS \geq 20 months	PFS 3-20 months	PFS \leq 3 months	<i>P</i> -value
T790M negative	36 (92.3%)	104 (93.7%)	25 (80.6%)	0.028
[0.01%-0.1%]	3 (7.7%)	1 (0.9%)	1 (3.2%)	
[0.1%-1.0%]	0	3 (2.7%)	1 (3.2%)	
[1.0%-10.0%]	0	3 (2.7%)	3 (9.7%)	
\geq 10.0%	0	0	1 (3.2%)	

PFS, progression-free survival ; VAF, variant allele frequency

Table 3. Univariate and multivariate survival analysis for VAF values of tumor pre-treatment *EGFR* T790M mutation.

Table 3A. Univariate and multivariate analysis for Progression-Free Survival (PFS)

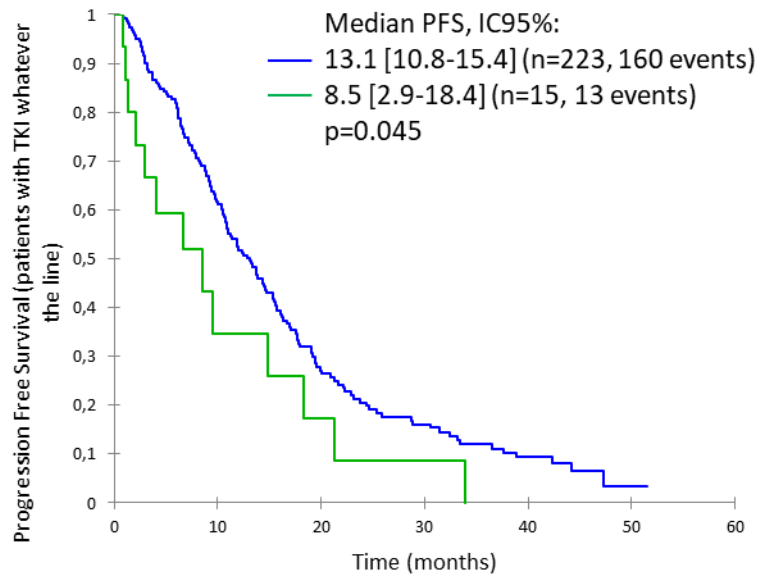
Characteristic	n	Univariate Analysis			Multivariate Analysis (n=225)		
		Hazard Ratio	95% CI	p	Hazard Ratio	95% CI	p
Gender							
Female	167	1	-	-			
Male	71	1.11	0.81-1.53	0.52			
Age							
<65 yrs	78	1	-	-	1	-	-
[65-80[yrs	126	0.58	0.42-0.80	0.001	0.51	0.36-0.71	<0.0001
>=80 yrs	34	0.60	0.37-1.00	0.05	0.51	0.30-0.87	0.01
Smoking history							
Current smokers	29	1	-	-			
Former smokers	60	0.67	0.39-1.16	0.15			
Never-smokers	144	0.68	0.41-1.11	0.12			
ECOG PS							
0/1	185	1	-	-	NS		
2/3/4	40	1.52	1.01-2.30	0.047			
TNM stage							
IV	208	1	-	-			
Relapse	30	1.14	0.72-1.79	0.58			
Histology							
Adenocarcinoma	203	1	-	-			
Larg Cell	3	1.42	0.35-5.75	0.63			
Squamous	1	1.10	0.70-1.73	0.68			
Other	31	3.30	0.46-23.86	0.24			
Mutation exon							
exon 21	109	1	-	-	1	-	-
exon 18	7	1.40	0.57-3.47	0.46	1.74	0.70-4.36	0.24
exon 19	122	0.57	0.42-0.78	0.0005	0.56	0.41-0.78	0.0006
T790M VAF							
Negative	223	1	-	-	1	-	-
[0.001%-0.01%[0	NA			NA		
[0.01%-0.1%[4	1.01	0.37-2.72	0.99	1.43	0.52-3.93	0.49
[0.1%-1.0%[5	1.26	0.40-3.97	0.69	1.51	0.46-4.92	0.49
[1.0%-10.0%[5	5.47	2.19-13.64	0.0003	3.62	1.43-4.92	0.007
≥10.0%	1	17.30	2.27-131.86	0.006	17.89	2.21-144.88	0.007

Table 3B. Univariate and multivariate analysis for Overall Survival (OS)

Characteristic	n	Univariate Analysis			Multivariate Analysis (n=218)		
		Hazard Ratio	95% CI	p	Hazard Ratio	95% CI	p
Gender							
Female	160	1	-	-	-		
Male	71	1.12	0.79-1.59	0.54			
Age							
<65 yrs	78	1	-	-	NS		
[65-80[yrs	122	0.73	0.52-1.04	0.08			
>=80 yrs	31	0.98	0.56-1.71	0.95			
Smoking history							
Current smokers	29	1	-	-	-		
Former smokers	60	0.70	0.40-1.22	0.21			
Never-smokers	137	0.70	0.42-1.17	0.17			
ECOG PS							
0/1	180	1	-	-	1	-	-
2/3/4	38	2.27	1.48-3.47	0.0002	2.13	1.51-3.00	<.0001
TNM stage							
IV	200	1	-	-	1	-	-
Relapse	31	1.60	1.03-2.49	0.04	1.86	1.17-2.94	0.009
Histology							
Adenocarcinoma	198	1	-	-			
Larg Cell	3	0.35	0.05-2.54	0.30			
Squamous	1	3.74	0.52-27.11	0.19			
Other	29	0.97	0.60-1.57	0.91			
Mutation exon							
exon 21	104	1	-	-	1	-	-
exon 18	7	1.40	0.56-3.47	0.47	0.98	0.35-2.76	0.97
exon 19	120	0.62	0.44-0.86	0.004	0.60	0.43-0.85	0.004
T790M VAF							
Negative	215	1	-	-	1	-	-
[0.001%-0.01%[0						
[0.01%-0.1%[4	1.96	0.72-5.32	0.18	2.85	1.03-7.83	0.04
[0.1%-1.0%[5	1.08	0.34-3.41	0.89	1.20	0.38-3.84	0.75
[1.0%-10.0%[5	3.29	1.34-8.12	0.01	2.83	1.13-7.07	0.03
≥10.0%	2	14.04	3.25-60.67	0.0004	19.14	4.35-84.26	<0.0001

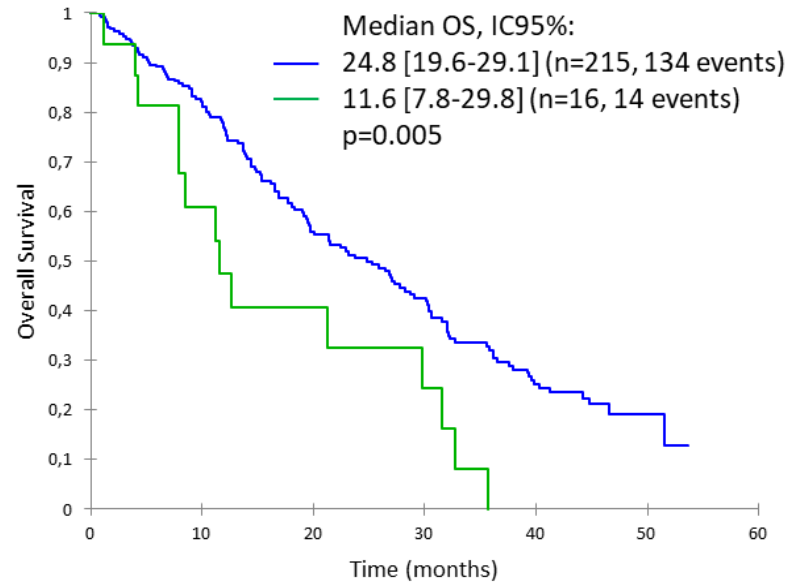
Figure 1

1A



	T790M negative		T790M positive				
Number at risk	223	114	39	21	10	1	0
T790 negative	223	114	39	21	10	1	0
T790 positive	15	4	2	1	0		

1B



	T790M negative		T790M positive				
Number at risk	215	160	87	63	33	7	0
T790 negative	215	160	87	63	33	7	0
T790 positive	16	9	5	3	0		

Figure 2

