

Highly sensitive droplet digital PCR method for detection of de novo EGFR T790M mutation in patients with Non-small cell lung cancer

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Abstract

Background This study was aimed to investigate the allelic relation between de novo T790M and concomitant sensitizing EGFR mutations in EGFR-TKIs naïve NSCLCs and to explore whether the formalin-fixed and paraffin-embedded (FFPE) materials affect the detection of de novo T790M mutation.

Methods 300 consecutive EGFR-TKI naïve NSCLCs who received surgical resection between January 2016 and June 2018 was retrospectively investigated. All the snap-frozen tumor tissues from 300 NSCLCs were screened by droplet digital PCR (ddPCR) for the detection of de novo T790M mutation. The allelic relation between de novo T790M mutation and concomitant sensitizing EGFR mutations was also investigated. Furthermore, we assessed de novo T790M mutation in paired FFPE specimens of 50 patients which included tumor tissues and paired normal lung tissues of the pretreatment NSCLCs to investigate whether FFPE materials affect the detection of de novo T790M mutation.

Results The de novo T790M mutation was observed in four patients which included one patient of single de novo T790M mutation and three patients of de novo T790M mutation coexisting with L858R mutation. The incidence of de novo T790M in pretreatment NSCLCs who harboring EGFR mutations was 2.9% (4/139). All the de novo T790M mutations were detected in cis with the concomitant L858R mutations for the three NSCLCs. Our ddPCR method demonstrated that the frequency of de novo T790M mutation were ranging from 0.1% to 0.5% among 90% (45/50) of the FFPE tumor samples and 92% (46/50) of the paired FFPE adjacent normal lung samples. The frequency of de novo T790M mutation in the paired snap-frozen samples were all below 0.1%.

Conclusion Our study demonstrated that most de novo T790M mutation were detected in cis with concomitant sensitizing mutations for pretreatment NSCLCs. Analytical cut-off of ddPCR assay for FFPE specimens should be validated carefully considering the possibility of FFPE-derived artificial gene mutations.

Background

Sensitizing mutations in the epidermal growth factor receptor (EGFR) gene are the most frequent oncogenic alterations in non-small cell lung cancer (NSCLC) presenting in approximately 10%-15% of Caucasians and 40%-50% of Asian patients with lung adenocarcinoma [1-4]. The most common sensitizing mutations in EGFR are in-frame deletions around the amino acids Leu Arg Glu Ala in exon 19 (19Del) and the L858R point-mutation in exon 21 (L858R), which are considered as classical EGFR mutations, accounting for approximately 85% of sensitizing EGFR mutations [5]. NSCLC patients with sensitizing EGFR mutations demonstrate improved objective responses and prolonged progression-free survival (PFS) under the treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib, afatinib and osimertinib [6-8].

The uncommon EGFR mutations (such as G719X, L861Q, S768I and T790M) comprise approximately 10% - 18% of all EGFR mutations [9, 10]. The emergence of T790M in exon 20 of EGFR has been demonstrated to be associated with acquired EGFR-TKI resistance which accounts for more than 50% of such cases [11]. Low frequency *de novo* T790M mutation has been detected in some EGFR-TKI treatment naïve NSCLC patients using several different highly sensitive methods [12, 13]. However, there were

discrepant results for the incidence of *de novo* T790M in different literatures with a wide range from 1% to 79% [12, 13]. The therapeutic efficacy of the third-generation EGFR-TKIs, such as osimertinib, is better than the earlier-generation EGFR-TKIs for the patients with concomitant *de novo* T790M and sensitizing EGFR mutations [14]. There is still controversy on whether the *de novo* T790M occurs occasionally or combines with the presence of other sensitizing EGFR mutations. It is important to identify the allelic relation between *de novo* T790M and concomitant sensitizing EGFR mutations among clinical specimens for the purpose to better understand the intrinsic mechanism of earlier-generation EGFR-TKIs resistance. However, literatures regarding this issue are limited and deserve further investigation. Moreover, it is still not clear whether the *de novo* T790M detection rate could be affected by formalin-fixed and paraffin-embedded (FFPE) samples which may cause artificial gene mutations (C-T or G-A transitions) [15]. Therefore, it is necessary to develop a reliable and stable platform with high sensitivity for the detection of *de novo* T790M in EGFR-TKI treatment naïve NSCLC patients.

Droplet digital PCR (ddPCR) is a new generation of PCR technique with high sensitivity and specificity for the detection of EGFR mutations [15, 16]. In the present study, Clinical specimens of 300 consecutive Chinese EGFR-TKI treatment naïve NSCLC patients were retrospectively collected. We investigated the prevalence and concomitant sensitizing EGFR mutations of *de novo* T790M mutation among them using a highly sensitive and specific ddPCR assay. Moreover, we investigated the allelic relation between *de novo* T790M and concomitant sensitizing EGFR mutations among the clinical specimens of EGFR-TKIs naïve NSCLC patients and explored whether the FFPE materials affect the detection of *de novo* T790M mutation.

Materials And Methods

Patients and specimen

Specimens of 300 consecutive Chinese EGFR-TKI treatment naïve NSCLC patients who received surgical resection at Peking University People's Hospital between January 2016 and June 2018 were retrospectively analyzed in this study. For the first 250 patients (Group A), only snap-frozen tumor tissues (stored at -80 °C) were analyzed. In the next 50 patients (Group B), both FFPE tumor tissues and snap-frozen tumor tissues (stored at -80 °C) were analyzed, and two types of the adjacent normal lung tissues were also analyzed simultaneously (**Figure 1**). This study was approved by the Ethics Committee of Peking University People's Hospital (Approved number: 2019 PHB 259-01).

ddPCR analysis

The ddPCR analysis was performed using human EGFR gene mutation detection kit (TargetingOne® Biotech. Co. Ltd. Beijing, China). The ddPCR platform system utilized for this evaluation was the TargetingOne® ddPCR System (TargetingOne® Biotech. Co. Ltd. Beijing, China). The TargetingOne® ddPCR System includes the Drop Maker and the Chip Reader. The Drop Maker is equipped with a microfluidic chip to quickly prepare the aqueous phase sample into a nanoliter drops. The number of

droplets is related to the sample volume, and about 50,000 - 60,000 droplets can be prepared from 30 microliters of aqueous phase sample. The Chip Reader uses the principle of laser confocal scanning and is equipped with a microfluidic chip to locate and identify nanoliter droplets accurately and quickly and obtain their fluorescence signal values. After Poisson statistical analysis, the absolute number of positive and negative droplets is provided to derive the exact concentration of the starting target nucleic acid molecule. The Chip Reader is compatible with TaqMan Hydrolysis Probes and EvaGreen Detection and has two channels which include the FAM (6-carboxyfluorescein) and the VIC channels.

The human EGFR gene mutation detection kit (TargetingOne[®]) can detect 42 common mutations in the 18 - 21 exons of the *EGFR* gene. There are six EGFR mutation assays in this kit, including 19Del, L858R, T790M, G719X, L861Q and S768I assays. The detection sensitivity was 0.1% - 0.5% mutation rate, depending on the type of sample and the type of assay. The reaction components were prepared as follows, 7.5 μ L of PCR reagent A (SuperMix), 7.5 μ L of PCR reagent B (primers and probes), and 20 - 50 ng of DNA extracted from FFPE tissues using QIAamp[®] DNA FFPE Tissue kit (Qiagen, Hilden, Germany) or from frozen tissues using TIANamp[®] Genomic DNA Kit (TIANGEN Biotech, Beijing, China). Sterilized water was used to make a volume of up to 30 μ L. Next, 30 μ L of digital PCR reaction mixture and 180 μ L of droplet generation oil (TargetingOne[®] Biotech. Co. Ltd. Beijing, China) were added into a droplet generation chip, and the droplet generation was performed using a Drop Maker following the manufacturer's instructions.

About 100 μ L of the resulting droplet emulsion were automatically transferred into an 8-strip PCR tube and amplified in a PTC-200 Thermal Cycler (Bio-Rad, CA). The PCR conditions were as follows: pre-denaturation at 95 °C for 10 minutes, amplification for 40 cycles, with denaturation at 95 °C for 30 seconds, annealing at 60 °C for 1 minute, ending at 12 °C for 10 minutes. After PCR, the 8-strip PCR tube containing the droplets was connected to a droplet detection chip, then the fluorescence signal of droplet was detected on Chip Reader. Finally, the data were subjected to Poisson distribution analysis using the TargetingOne[®] ddPCR software to obtain the target DNA copy number in the samples.

Statistical analysis

The statistical analysis was performed with Statistical Product and Service Solutions (SPSS) software versions 20.0 (2011; IBM, Armonk, NY, USA). Differences were compared using a *t* test for continuous clinicopathological variables, and *Chi-square* test or *Fisher's exact* test for categorical mutations and clinicopathological variables. *P* value < 0.05 was considered statistically significant.

Results

Patient characteristics

The median age of the cohort was 62 years old (ranging from 35 to 88 years old), which including 171 males and 129 females. Seventy-three (24.3%) were smokers, and more than 50% patients were stage I

NSCLC. There were 224 (74.7%) adenocarcinomas, 72 (24.0%) squamous cell carcinomas and 4 (1.3%) adeno-squamous carcinomas. EGFR mutations were mainly distributed in non-smoker, female patients and adenocarcinomas. The baseline characteristics of the 300 patients were listed in **Table 1**.

The condition of EGFR mutations in snap-frozen tumor tissue

Snap-frozen tumor tissues from 300 EGFR-TKI naïve NSCLC patients were screened by ddPCR for the detection of EGFR mutations including 19Del, L858R, T790M, G719A, S768I and L861Q. EGFR mutations were observed in 139 of 300 (46.3%) patients. Single EGFR mutations were detected in 134 patients, including 49 (16.3%) patients with 19Del, 79 (26.3%) patients with L858R, 4 (1.3%) patients with L861Q, 1 (0.3%) with T790M, and 1 (0.3%) with G719A. Concurrent EGFR mutations were detected in 5 (2.3%) patients including three patients with EGFR L858R+T790M mutations, one patient with EGFR G719A+L861Q mutations and one patient with EGFR G719A+S768I mutations (**Table 2**). All the uncommon EGFR mutation were detected in patients with adenocarcinoma. Patients characteristics and pathologic stages were listed in table 3.

De novo EGFR T790M mutation was observed in 4 (4/300, 1.3%) patients with an incidence of 2.9% (4/139) in the EGFR mutated cohort, including one patient with single *de novo* T790M mutation and three patients harboring *de novo* T790M mutation coexisting with L858R mutation which suggested that the *de novo* T790M was more likely to coexist with EGFR L858R mutation (**Table 3**).

Evaluation of the allelic relation between de novo T790M and sensitizing EGFR mutations in NSCLC specimens

Furthermore, we investigated the allelic relation between *de novo* T790M and concomitant sensitizing EGFR mutations. In our study, concomitant occurrence of *de novo* T790M and L858R was detected in three individuals and no patient was found to have a *de novo* T790M combined with 19Del. Total RNA was obtained from the three patients with *de novo* T790M combined with L858R. A reverse transcription (RT) ddPCR-based method was designed to evaluate the allelic relation between the two EGFR mutations. Analysis was performed with the mutation-specific probes which were designed as upstream primers of the T790M and the probe targeting *de novo* T790M of EGFR (labeled with FAM) combined with downstream primers of the L858R and the probe targeting L858R of EGFR (labeled with VIC). Double positive signals for the *de novo* T790M and L858R of EGFR were detected among the three NSCLC patients which demonstrated that all the *de novo* T790M mutations were present together with the EGFR L858R mutations on the same allele (**Figure 2**).

Influence of FFPE materials on the detection of de novo T790M mutation

For the 50 patients in group B, FFPE sample of tumors and their adjacent normal lung tissues were also analyzed. Droplet digital PCR was used for the detection of EGFR mutations including 19Del, L858R and T790M. Together with the detection results of snap-frozen samples from the same cohort, EGFR mutations were detected in 26 NSCLC tumor samples regardless of the specimen type. And most patients

(24/26) with common sensitizing EGFR mutations had concordant mutations between the snap-frozen tumor samples and FFPE tumor tissue samples. Surprisingly, *de novo* T790M mutation was detected in 90% (45/50) of the FFPE tumor samples and 92% (46/50) of the paired FFPE adjacent normal lung samples with a frequency of 0.1%-0.5% using our ddPCR method (**Figure 3**). However, the frequency of *de novo* T790M mutation in the paired snap-frozen samples were all below 0.1% which were identified as *de novo* T790M-negative according to the identifying standard for frozen samples.

Discussion

The emergence of EGFR T790M mutation is a major cause of acquired resistance to first generation EGFR-TKIs for NSCLC patients harboring sensitizing EGFR mutations. Moreover, several studies suggested that the *de novo* T790M mutation was present in a minor subgroup among EGFR-TKI treatment naïve NSCLC patients [17, 18]. It is reported that the incidence of *de novo* T790M mutation could be up to about 79% among sensitizing mutation-positive NSCLC patients before EGFR-TKI treatment using highly sensitive methods, such as ddPCR or colony hybridization, and most of the *de novo* T790M mutations were below 0.1% in their frequency [13, 17]. But studies from other groups reported that they detected *de novo* T790M mutations in 0.5-1% EGFR-TKI naïve NSCLC patients by ARMS or targeted sequencing with sensitivity of about 1% [12,14]. The great difference of reported incidence of *de novo* T790M mutation may come from the detecting sensitivity of the methods. This hypothesis was supported by the study from Inukai *et al* in which only one T790M mutant case was detected by the direct sequencing, while 9 additional cases among 280 cases were detected using mutant-enriched PCR (ME-PCR, sensitivity of 0.1%) [18]. However, the accurate incidence of *de novo* T790M is still largely unknown. Further, the influence of FFPE sample on the detection rate of T790M, as well as the allelic relationship of *de novo* T790M and concurrent sensitizing mutations have not been well proved. In this study, snap frozen tumor tissue from 300 consecutive Chinese EGFR-TKI naïve NSCLC patients were tested using ddPCR to investigate the prevalence of *de novo* T790M mutation. The incidence of *de novo* T790M in pretreatment NSCLC patients who harboring EGFR mutations was 2.9% (4/139) with adenocarcinoma in our cohort, which was consistent with Ye's report using ME-PCR with sensitivity of 0.1%.

For the purpose to detect the accompanying mutations coexisted with *de novo* T790M mutation, two common sensitizing EGFR mutations (L858R and 19Del) and three most frequent uncommon EGFR mutations (G719A, S768I and L861Q) were detected simultaneously. Consistent with previous studies [12, 14, 20], we found that most *de novo* T790M mutation coexist with L858R mutation (75%, 3/4). *De novo* T790M mutation could also co-exist with 19 Del, as well as other uncommon EGFR mutations [12, 20]. The reason we did not find co-existing *de novo* T790M and 19 Del in our study perhaps because of the small sample size and rare *de novo* T790M cases. However, it was reported that acquired T790M mutation always coexist with 19Del [20-22]. Tian's study [22] reported that the ratio of allele frequency (Relative allele frequency, RAF) of the T790M mutation to the EGFR sensitizing mutation was different between the *de novo* and acquired T790M mutations (86.1% vs. 22.3%, $P < 0.0001$). Hata *et al* [23] reported that the T790M mutations could occur both by the selection of pre-exist T790M clones and the

genetic evolution from drug tolerant cells, and the two types of T790M positive cells developed at different times and showed differential response to the EGFR inhibition which suggested that they might have different molecular characteristics. The intrinsic mechanism of the different trends for the association between T790M mutations (*de novo* or acquired) and sensitizing EGFR mutations remains unclear.

The only study to investigate the allelic relation between *de novo* T790M and the concurrent sensitizing EGFR mutations in the treatment naïve NSCLC patients to date was reported by Hidaka *et al* [24], and only one such case was reported in their study. In our study, we investigated the allelic relation between *de novo* T790M and concurrent EGFR L858R mutations among the three clinical specimens from EGFR-TKI naïve NSCLC in our cohort. Our result further conformed that all *de novo* T790M mutations are present on the same EGFR allele with the sensitizing EGFR mutations in the pretreatment NSCLC patients.

Theoretically, the *in cis* compound mutations of *de novo* T790M and L858R mutations might affect the binding of first- or second- generation EGFR-TKIs to the ATP-binding site of EGFR. For the NSCLC patients with *de novo* T790M and sensitizing EGFR mutation, the use of earlier-generation EGFR-TKIs would lead to a decrease of sensitive target cells and an enrichment of resistant double-mutated tumor cells [4, 24].

The influence of FFPE sample on the detection rate of T790M was another concern when evaluating the prevalence of *de novo* T790M. Watanabe *et al* [17] reported that they detected *de novo* T790M mutations in 79.9% (298/373) FFPE tumor specimens of pretreatment NSCLC patients, using ddPCR with a sensitivity of 0.001%. In Iwama's study [25], the prevalence of *de novo* T790M mutation was 100% (25/25) among the pretreatment FFPE tissue specimens by ddPCR technique with a sensitivity of 0.01%. However, such a prevalence of *de novo* T790M mutation might be due to the false-positive rate caused by highly sensitive methods in clinical molecular testing. Ye *et al* [15] demonstrated that the incidence of *de novo* T790M mutation in FFPE specimens was much higher than that in paired frozen samples, and the artificial T790M mutation could be detected in 48.8% (16/33) FFPE adjacent normal samples of EGFR-TKI naïve NSCLC patients, using mutant-enriched PCR method (ME-PCR). However, there was no subsequent studies to provide more evidence to clarify this issue. In the present study, we assessed the *de novo* T790M in 50 pairs of frozen and FFPE tumor and adjacent normal tissues specimens of EGFR-TKI naïve NSCLC patients, using ddPCR with sensitivity of 0.1%. Our ddPCR method demonstrated that the frequency of *de novo* T790M mutation were ranging from 0.1% to 0.5% among more than 90% of the FFPE tumor and normal lung samples. Meanwhile, the frequency of *de novo* T790M mutation were all below 0.1% in the paired snap-frozen samples, which was consistent with Ye's report. Therefore, analytical cut-off of ddPCR assay for FFPE specimens should be validated carefully before their application on clinical testing considering the possibility of FFPE-derived artificial gene mutations.

Many studies have demonstrated unfavorable outcome in patients with co-existence of *de novo* T790M mutation who were treated with first generation TKIs [14, 22, 26]. However, a previous meta-analysis by Liu *et al* [27] indicated that the *de novo* T790M mutation did not affect the PFS or the OS in NSCLC patients who harbored sensitizing EGFR mutations and were treated with EGFR-TKIs. An *in vitro* experiment [28] reported that the cell lines of NSCLC harboring T790M and L858R mutations exhibited

increased phosphorylated EGFR protein expression compared to single L858R mutation which was linked to the resistance to first- and second-generation EGFR inhibitors. It is presumed that the sensitivity to the earlier-generation EGFR-TKIs would be affected by the abundance of *de novo* T790M mutation among the NSCLC patients. Therefore, the negative results of the Liu's meta-analysis might come from the results of some studies with ultra-low allele frequency of *de novo* T790M. Thus, it is necessary to use appropriate methods, select optimal cut off values, and confirm positive results by an alternative method in clinical molecular diagnosis.

Some previous studies [12, 27] reported that the patients with *de novo* T790M mutation could benefit from osimertinib as the first-line treatment. The FLAURA study [29] suggested that the median PFS for the sensitizing EGFR mutation positive NSCLCs could be 18.9 months by the initial using of osimertinib. It seemed that to use osimertinib as first-line treatment for sensitizing EGFR mutation positive NSCLCs did not significantly improve the PFS than that of sequential use of earlier-generation EGFR-TKIs followed by third-generation EGFR-TKIs. However, the planned final analysis of the FLAURA trial [30] showed that to use osimertinib as first-line treatment for the sensitizing EGFR mutation positive NSCLCs could have a better median OS than that of first-generation EGFR-TKIs (38.6 vs. 31.8 months, $P = 0.046$). The clinical benefit might be attributed to the existence of *de novo* T790M mutation and the central nervous system metastases, and various different potential resistance mechanisms which involved drug pressure or genetic evolution.

There are some limitations to this study. First, the selection bias could not be avoided considering the retrospective nature of the study. Second, the number of patients with sensitizing EGFR mutations were relatively small and only 3 cases were detected with concurrent *de novo* T790M and sensitizing EGFR mutations. Third, most of our patients were early-stage NSCLCs and lacking treatment results with TKIs. Fourth, the spatial heterogeneity of the tumor might have an influence on the detection of the *de novo* T790M mutation.

Conclusions

In conclusion, we tested the prevalence of *de novo* T790M mutation in NSCLC using sensitive ddPCR method and snap frozen tumor tissue, which would offer relative accurate results. We reported the allelic relationship of *de novo* T790M and concurrent sensitizing mutations in three cases, which was the largest cohort to date with great importance. And our study also evaluated the influence of FFPE specimen on the detection rate of T790M, which could be considered in clinical molecular diagnosis.

List Of Abbreviations

NSCLC: Non-small cell lung cancer;

ddPCR: Droplet digital PCR;

EGFR: Epidermal growth factor receptor;

EGFR-TKIs: Epidermal growth factor receptor tyrosine kinase inhibitors;

FFPE: Formalin-fixed and paraffin-embedded;

PFS: Progression-free survival.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

FY, YG and JW contributed to the design, overall planning of the study. XW, XL contributed to the data verification, paper writing, and offered some thoughts for the study's design. XW, HG, LZ and ZP contributed to collect and analyze the data. All authors read and approved the final manuscript.

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Availability of data and materials

Authors can provide all of datasets analyzed during the study on reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Peking University People's Hospital (Approved number: 2019 PHB 259-01).

Consent for publication

All authors have approved the submitted manuscript.

Competing interests

The authors declare that they have no competing interests.

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Table 2. A comprehensive view of the EGFR mutations.

EGFR mutation	Overall population (N=300)	Adenocarcinoma (N=224)	Non-adenocarcinoma (N=76)
EGFR L858R	79 (26.3%)	75	4
EGFR T790M	1 (0.3%)	1	0
EGFR L858R+T790M	3 (1.0%)	3	0
EGFR G719A	1 (0.3%)	1	0
EGFR L861Q	4 (1.3%)	4	0
EGFR G719A+L861Q	1 (0.3%)	1	0
EGFR G719A+S768I	1 (0.3%)	1	0
Total	139 (46.3%)	134 (59.8%)	5 (6.6%)

EGFR, epidermal growth factor receptor.

Table 3. The characteristics of the patients with uncommon EGFR and *de novo* T790M mutations.

No.	Gender	Histology	status	Smoking	status	Stage	T790M	L858R	19Del	G719A	S768I	L861Q
No. 1	F	Ade	No	III A	-	-	-	29.48%	32.60%	-	-	-
No. 2	F	Ade	No	III A	-	-	-	-	-	-	-	30.04%
No. 3	F	Ade	No	I A	-	-	-	2.01%	-	-	-	-
No. 4	F	Ade	No	I A	-	-	-	-	-	-	-	28.87%
No. 5	F	Ade	No	I B	-	-	-	17.30%	-	-	-	9.17%
No. 6	F	Ade	No	I I B	-	-	-	-	-	-	-	32.29%
No. 7	F	Ade	No	I A	-	-	-	-	-	-	-	12.89%
No. 8	F	Ade	No	I A	7.7%	8.3%	-	-	-	-	-	-
No. 9	F	Ade	No	I B	7.7%	7.2%	-	-	-	-	-	-
No. 10	F	Ade	No	I B	5.6%	-	-	-	-	-	-	-
No. 11	M	Ade	No	I I B	24.49%	43.37%	-	-	-	-	-	-

F, Female; M, Male; Ade, Adenocarcinoma.

Figures

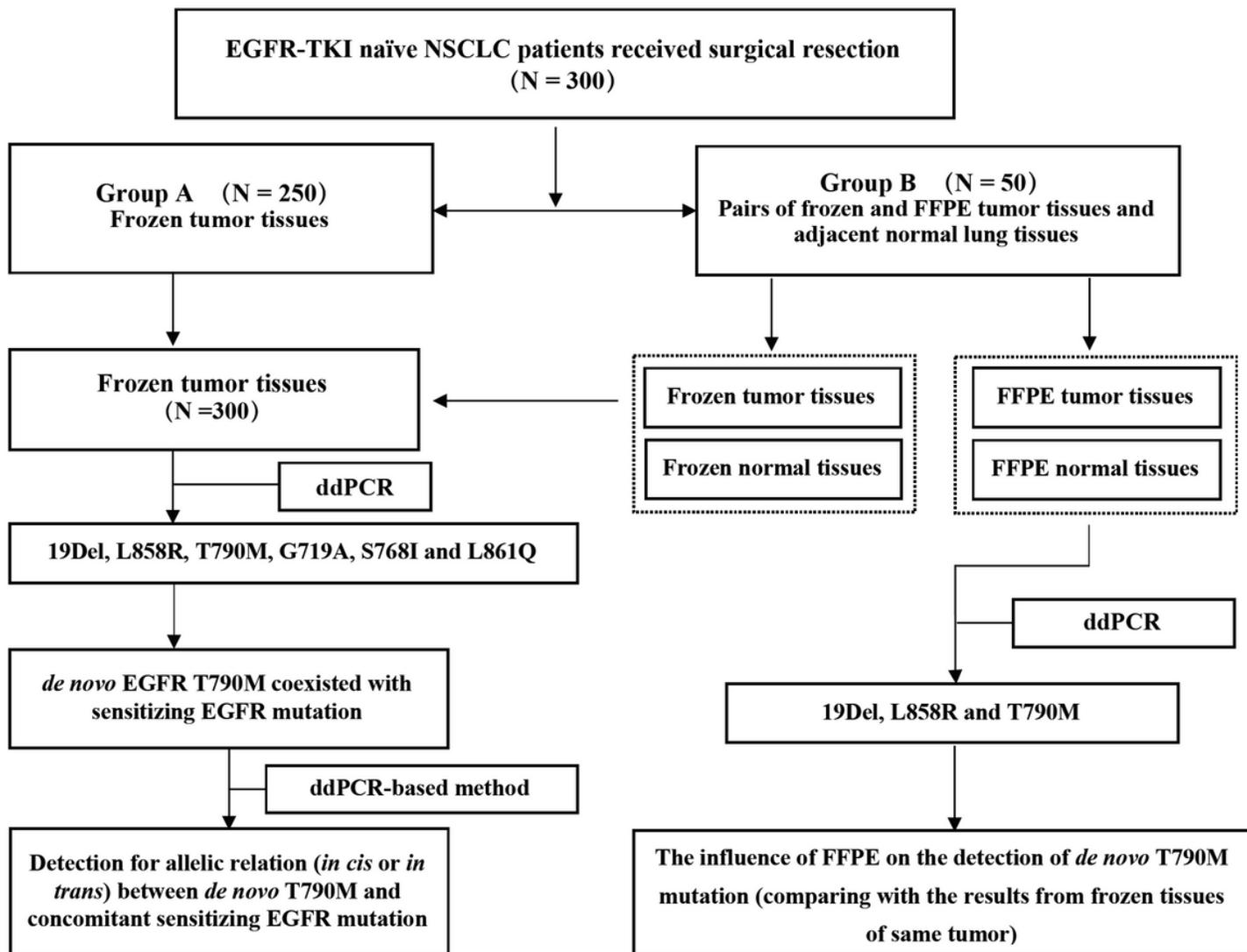


Figure 1

The flowchart of the study design.

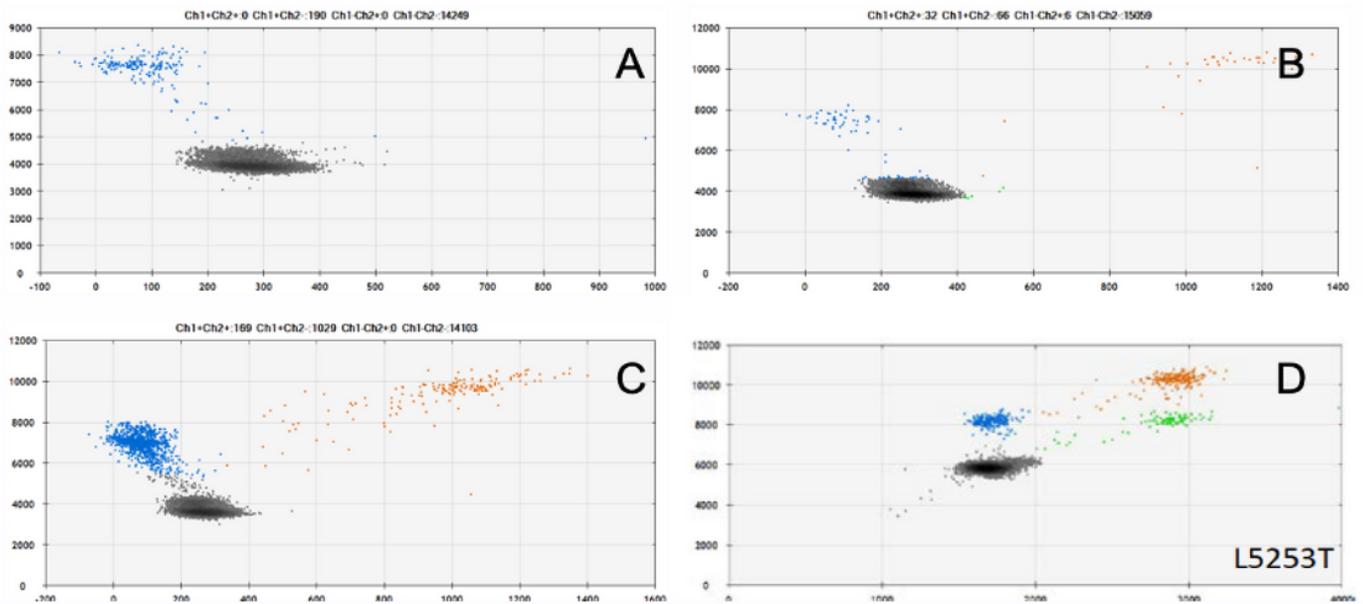


Figure 2

(A) Non-specific signals of FAM were shown for the NSCLC cell lines with wild type EGFR. (B, C, D) Three clinical pretreatment NSCLC patients with concomitant de novo T790M and L858R mutations in which a ddPCR method was performed to detect the allelic relation between the two mutations, double positive signals for the de novo T790M (FAM labeled) and L858R of EGFR (VIC labeled) were shown in the B, C and D. For the ddPCR results, the FAM and VIC negative signals were indicated as black color. The signals of wild-type EGFR L858R and wild-type T790M mutations were depicted as blue color. The signals of EGFR L858R mutation positive were depicted as green color. Double positive signals for the de novo T790M with in cis L858R mutation were depicted as orange color.

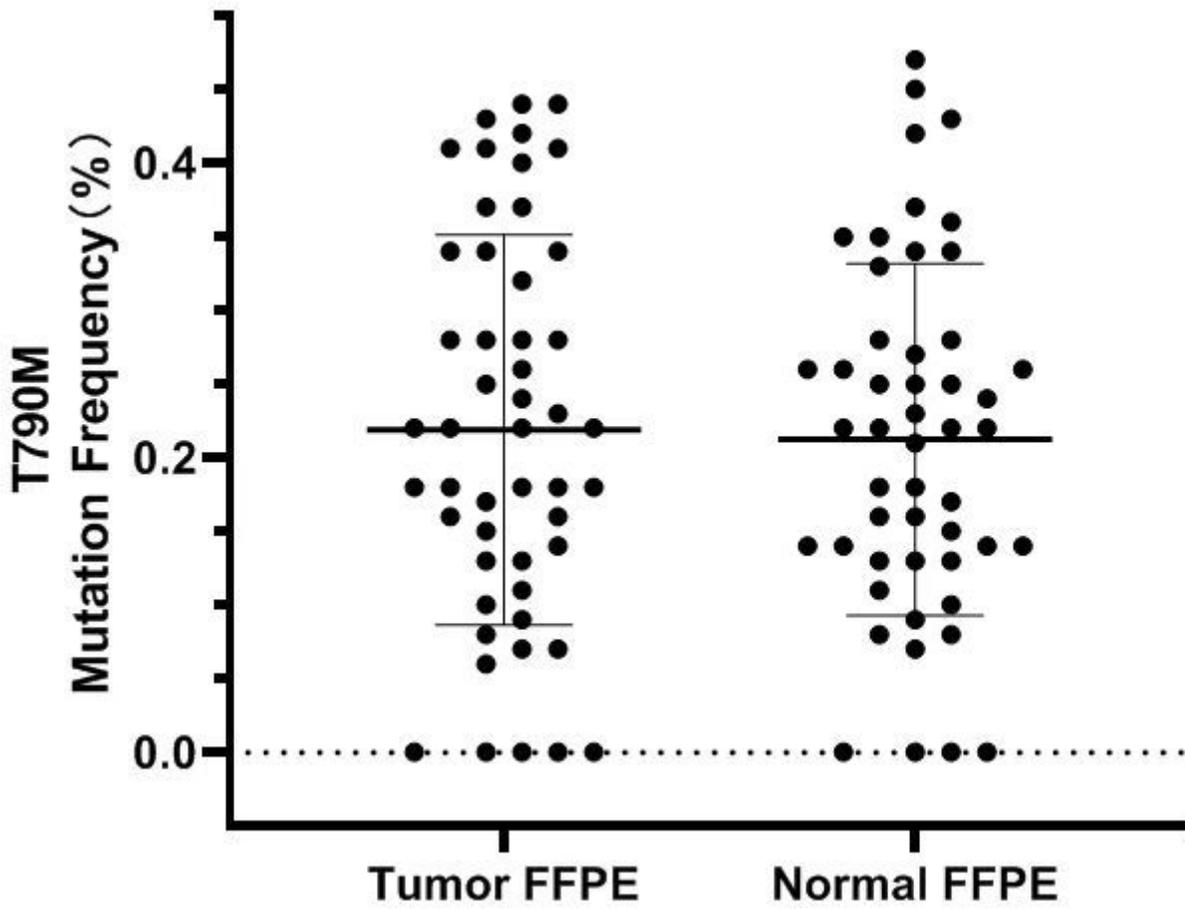


Figure 3

The de novo T790M mutant abundance in the FFPE tumor tissues samples and FFPE adjacent normal lung samples.