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ORIGINAL ARTICLE

Absence of *EGFR* C797S Mutation in Tyrosine Kinase Inhibitor-Naïve Non–Small Cell Lung Cancer Tissues

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Abstract
EGFR tyrosine-kinase inhibitors (TKIs) are used as targeted therapeutics for the treatment of advanced non–small cell lung cancer (NSCLC) with EGFR-activating mutations. EGFR C797S is common causes of acquired resistance to third-generation TKIs. There is wide-spread opinion that resistance-conferring mutation present even in a small proportion of cancer cells before the start of therapy could potentially predict poor response to a targeted drug. In our study, we tested whether C797S can be found in previously untreated NSCLCs. We analyzed DNA samples extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue sections of 470 lung adenocarcinoma patients, including 235 samples with activating EGFR mutations. Screening was performed using highly sensitive droplet digital PCR assay. No tumor samples with baseline C797S were identified. C797S does not occur in TKI-naïve NSCLCs and provide evidence that screening for this mutation before TKIs administration may not be necessary.

Keywords Non-small cell lung cancer · EGFR · C797S · Osimertinib · Droplet digital PCR

Introduction

Epidermal growth factor receptor (EGFR) is an important drug target for non–small cell lung cancer (NSCLC) therapy. The standard care for advanced NSCLC patients harboring activating *EGFR* gene mutations is treatment with tyrosine-kinase inhibitors (TKIs). TKIs provide longer progression-free survival as compared to platinum-based chemotherapy and less commonly cause severe or life-threatening adverse events. However, almost all patients treated with first-generation reversible TKIs (erlotinib and gefitinib) experience disease progression within 9–14 months of therapy [1–3]. The leading cause of acquired resistance is occurrence of the secondary *EGFR* T790 M mutation, which accounts for about 60% of TKI-relapsed NSCLCs. Second-generation irreversible TKIs, such as afatinib, were initially anticipated to overcome T790 M-mediated resistance. Albeit these molecules can bind EGFR^{T790M}, they are also active against physiologically important wild-type EGFR. As a result, drug doses needed to effectively inhibit T790 M-mutated EGFR exceed the highest doses tolerated by patients, so clinical application of second-generation TKIs is limited [2, 4]. Third-generation selective TKIs spare wild-type EGFR and have good potency against NSCLCs bearing both activating and T790 M *EGFR* mutations. The first globally approved third-generation TKI for the treatment of EGFR^{T790M} NSCLC patients is osimertinib. Moreover, this drug has been recently shown to be superior to erlotinib and gefitinib as the first-line therapy for previously untreated patients [5]. Several other third-generation TKIs are now at different stages of development, like rociletinib, olmutinib, nazartinib, naquotinib, avitinib [6–10]. Unfortunately, resistance to all that drugs inevitably emerges, and one of the most common reasons of treatment failure is the tertiary *EGFR* C797S mutation [3, 11]. No therapeutic agent has still been developed that can circumvent C797S-mediated resistance, although several promising compounds are now discovered [3, 12].

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TKI resistance-conferring mutations can be detected in pre-treatment lung cancers. Previous studies reported the presence of T790 M in NSCLCs before the exposure to TKI, and demonstrated its potential clinical importance [13, 14]. Yu et al. observed low response rate to erlotinib and short progression-free survival in patients with baseline T790 M found by routine clinical assays, e.g. Sanger sequencing [14]. The proposed mechanism for poor response is selective proliferation of a T790 M-positive tumor cells under the first-generation TKIs treatment, so that even a small proportion of such cells can eventually drive TKI resistance [13]. Several studies also report rarely occurring inherited T790 M to be a risky factor for lung cancer [15–17]. It is possible that the same mechanism may impair the response to third-generation TKIs, and C797S present in inherited state or in a small fraction of pre-treatment NSCLC cells can underlie subsequent resistance development. Data on C797S presence in TKI-naïve NSCLCs are limited. The single study by Thress et al. reported zero frequency of C797S in 15 subjects with activating *EGFR* mutations before osimertinib therapy [11]. More research is needed to elucidate occurrence of baseline C797S. In the present study, we aimed to analyze C797S frequency in pre-treatment NSCLC tissue samples from 470 patients using in-house highly sensitive droplet digital polymerase chain reaction (ddPCR) assay.

Materials and Methods

Study Sample and DNA Extraction

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue sections using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) in accordance with manufacturer's protocol. FFPE sections were obtained from 470 lung adenocarcinoma patients that have been operated in 14 regional cancer centers across the Siberian and the Far Eastern Federal Districts of Russia. None of patients have received TKI treatment before sampling. Details of the studied group are given in Table 1.

Table 1 Descriptive characteristics of patients with lung adenocarcinoma ($N = 470$) and the prevalence of *EGFR* gene mutations

Parameter	Value
Women, number of patients (%)	146 (31.0%)
Men, number of patients (%)	324 (69.0%)
Age, years, median (lower quartile, upper quartile)	62 (57, 67)
T*, number of patients (%)	T1: 72 (15.2%)
	T2: 177 (37.7%)
	T3: 105 (22.4%)
	T4: 110 (23.3%)
	Tx: 6 (1.3%)
N*, number of patients (%)	N0: 103 (22.0%)
	N1: 93 (19.7%)
	N2: 179 (38.1%)
	N3: 48 (10.3%)
	Nx: 46 (9.9%)
M*, number of patients (%)	M0: 255 (54.3%)
	M1: 215 (45.7%)
EGFR gene mutations	Prevalence
Exon 19 deletions, number of patients (%)	114 (24.2%)
L858R, number of patients (%)	114 (24.2%)
Exon 20 insertions, number of patients (%)	4 (0.8%)
G719X, number of patients (%)	2 (0.4%)
L861Q, number of patients (%)	0 (0.0%)
S768I, number of patients (%)	3 (0.6%)
T790 M, number of patients (%)	0 (0.0%)
C797S, number of patients (%) – screened by ddPCR§	0 (0.0%)

* staging according to the TNM classification of malignant tumors (7th edition). T, size and extent of the primary tumor; N, degree of spread to regional lymph nodes; M, presence of distant metastasis, number of patients. EGFR mutations with the exception of C797S were tested using therascreen EGFR RGQ PCR Kit, Version 1 (Qiagen, Germany) according to manufacturer's protocol

§ LoD = 0.01%

Detection of Common EGFR Gene Mutations

Common *EGFR* mutations were analyzed using therascreen EGFR RGQ PCR Kit, Version 1 (Qiagen, Germany) according to manufacturer's instructions. Amplification procedure was conducted using CFX96 Thermal Cycler (Bio-Rad, USA). The manufacturer-reported limits of detection (LoD) were as follows: 7.02% for T790 M, 1.26% for L858R, 1.64% for exon 19 deletions, 0.50% for L861Q, 5.43% for G719X, 1.37% S768I, and 2.03% for exon 20 insertions (for the multiple assays, the highest LoD is indicated).

Droplet Digital PCR Assay for C797S

C797S mutation was analyzed by a ddPCR assay performed with the QX100 Droplet Digital PCR system (Bio-Rad, USA). Primers and probes sequences were obtained from the previously published study by Thress et al. [11]. The 20 µL ddPCR reaction mixture contained ddPCR master mix (Bio-Rad, USA), 0.9 µM primers, 0.25 µM probes, and 40–50 ng of tested DNA. The entire reaction mixture together with 70 µL of droplet generation oil (Bio-Rad, USA) was loaded into a disposable plastic cartridge (Bio-Rad, USA) and placed in the droplet generator. After processing, droplets obtained from each sample were transferred to a 96-well PCR plate (Eppendorf, Germany). Amplification was carried out using T100TM Thermal Cycler (Bio-Rad, USA). PCR thermal cycling conditions were as follows: DNA polymerase activation at 95 °C for 10 min followed by 45 cycles of PCR amplification (94 °C for 30 s and 57 °C for 60 s), and 98 °C for 10 min, 2 °C/s ramp rate at all steps. PCR-positive and PCR-negative droplets were counted with the QX100 Droplet Reader. The data acquired were analyzed with QuantaSoft software (Bio-Rad, USA) (Fig. 1).

Positive Controls, Limits of Blanks, and Limits of Detection

Plasmids carrying EGFR 797S alleles served as positive controls and were used to assess method sensitivity. Plasmids were constructed by overlapping PCR with a human genomic DNA template and the following combinations of primers: EGFR-20ex-F/C797S-C-R, EGFR-20ex-R/C797S-C-F (for p.C797S c.2390G > C), EGFR-20ex-F/C797S-A-R, EGFR-20ex-R/C797S-A-F (for p.C797S c.2389 T > A). Sequences of primers are listed in Table 2. The resultant amplified fragments were fused via the second round of PCR with EGFR-20ex-F/R primers, digested with BamHI/XhoI endonucleases, and ligated with pMTL22 vector (BamHI/XhoI). Finally, plasmids containing mutant alleles (p.C797S c.2390G > C, and p.C797S c.2389 T > A) were purified, sequenced, and quantified using NanoDrop Lite A4 spectrophotometer (Thermo Fisher Scientific, USA).

Limit of blank (LoB) and limit of detection (LoD) were determined as previously described [18]. The estimated values of LoB and LoD were 0.003% and 0.01% for both C797S variants.

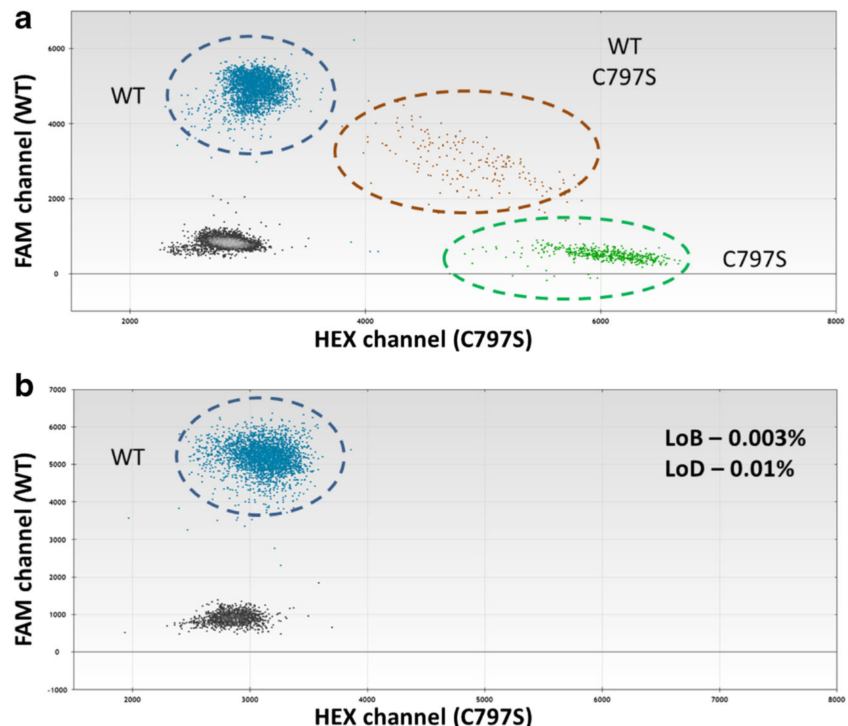

Fig. 1 ddPCR plots visualized with QuantaSoft software (Bio-Rad, USA). Blue cluster depicts droplets containing wild-type *EGFR* (no 797S), green cluster corresponds to droplets with C797S-mutated *EGFR*, brown cluster indicates droplets with both wild-type and mutant alleles, and black dots depict empty droplets. **a** C797S-positive plasmid control; **b** C797S-negative NSCLC sample

Table 2 Primers and probes for EGFR C797S and T790 M analysis

Primer/probe	Sequence
Primers for construction of positive plasmid controls	
EGFR-20ex-F (common primer)	5′-CACACTCGAGATGCGAAGCCACACTGAC-3′
EGFR-20ex-R (common primer)	5′-ACCGCATGTGAGGATCCT-3′
C797S-C-F (c.2390G > C mutation)	5′-CTTCGGCTCCCTCCTGG-3′
C797S-C-R (c.2390G > C mutation)	5′-CCAGGAGGGAGCCGAAG-3′
C797S-A-F (c.2389 T > A mutation)	5′-CTTCGGCAGCCTCCTGG-3′
C797S-A-R (c.2389 T > A mutation)	5′-CCAGGAGGCTGCCGAAG-3′
Primers and probes for ddPCR assay	
797-F	5′-GCCTGCTGGGCATCTG-3′
797-R	5′-TCTTTGTGTTCCCGGACATAGTC-3′
C797S-WT	5′-FAM-TTCGGCTGCCTCCTG-BHQ1–3′
C797S-Mut-A (c.2389 T > A mutation)	5′-HEX-TTCGGCAGCCTCC-BHQ2–3′
C797S-Mut-C (c.2390G > C mutation)	5′-HEX-CTTCGGCTCCCTCCTG-BHQ2–3′

*sequences were obtained from the study by Thress et al. [6]

Results

The prevalence of *EGFR* gene mutations in TKI-naïve NSCLC samples is presented in Table 1. Half of enrolled patients (235) were positive for any of *EGFR* activating mutations. Among them, 228 patients carried sensitizing for TKIs treatment mutations: 114 with deletions in 19 exon, and 114 with L858R. Other 7 patients carried rare *EGFR* mutations: 2 – G719X, 3 – S768I, and 4 – exon 20 insertion, which are associated with poor response to first- and second-generation TKIs. It should be noted, that our sample panel was intentionally enriched with *EGFR*-mutated specimens, while reported frequency of *EGFR*-activating mutations in Russia is about 18% [19].

No patients had baseline C797S resistance mutations. Given a high sensitivity of the ddPCR assay used to screen for C797S, we can conclude that either NSCLC tissue samples did not contain any $EGFR^{C797S}$ subclones, or C797S mutant allele frequency was extremely low (less than our limit of detection of 0.01%). Thus, our study provides no evidence for the presence of C797S in TKI-naïve NSCLC and does not support the hypothesis that resistance to third-generation TKIs could arise from baseline C797S.

Discussion

EGFR harboring several activating mutations, such as L858R and deletions in exon 19, can be selectively inactivated by specific tyrosine-kinase inhibitors. However, resistance occurs unavoidably during the treatment of tumor. For the first generation of EGFR inhibitors (erlotinib, gefitinib) the most common reason of resistance is acquired T790 M mutation in *EGFR* gene. Several molecules has been developed to overcome EGFR T790 M-related resistance, among them osimertinib was the first globally approved. Like in the case of first generation anti-EGFR drugs patients treated with osimertinib invariably progress. Concrete mechanisms of resistance to osimertinib have yet to be thoroughly studied, whereas recent studies provide a glimpse into the problem [20–25]. Thus, amplification of *MET*, mutation in *EGFR*, *BRAF*, *KRAS*, *NRAS*, *PIK3CA* and several other gene alterations have been reported in samples from osimertinib-resistant tumors. Among them tertiary C797S mutation in *EGFR* with proved mechanism of action is one of the most common, being found in 16% of patients with developed resistance.

Intratumoral heterogeneity can be the root cause of therapy resistance. Tumor may continue to progress due to selective outgrowth of a minor subpopulation of cells that had acquired survival mechanisms [4, 12]. In some cases, drug resistance-conferring mutations could be present stochastically in tumor cells prior to cancer therapy [13]. Several cases of inherited T790 M were also reported too [15–17]. Germline T790 M, as well as activating mutation R776H [26, 27], are believed to increase a risk for lung cancer development. Accordingly to the Exome Aggregation Consortium (ExAc) data, inherited T790 M is rare mutation with minor allele frequency 0.00004, while germline C797S was not found. Up to date the possible presence and frequency of C797S in TKI-naïve patients is unknown, making difficult to comprehend origin of the osimertinib resistance. If frequency of primary C797S is traceable, testing of C797S before osimertinib usage should become obligatory to prevent tumor response. For better assessment sensitive testing method is advantageous due to somatic nature of C797S mutation in lung cancer; digital PCR methods are proved to be sensitive enough for the detection of single DNA molecules bearing mutations.

In our study, we screened for the presence of C797S in patients who had not previously been treated with any TKI. We used a highly sensitive detection method and found no C797S-mutated alleles in 470 TKI-naïve NSCLC samples. It can be speculated that stand-alone C797S mutation is at least

neutral or even detrimental for malignant cells without selective pressure when osimertinib is applied. Basing on our results, we can suppose that C797S may be acquired only after osimertinib administration, so testing for C797S in pre-treatment NSCLC samples would not be useful for predicting the response to third-generation TKIs.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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