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Improved detection of T790M Mutation in the *EGFR* gene at the Institute for Oncology and Radiology of Serbia: A comparative study of dPCR and qPCR technologies over two years

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Abstract

The T790M mutation in the *EGFR* gene is a critical biomarker of resistance in patients undergoing EGFR tyrosine kinase inhibitor (TKI) therapy. Accurate and efficient detection of this mutation is essential for guiding treatment decisions in non-small cell lung cancer (NSCLC) patients. This study, conducted over two years at the Institute for Oncology and Radiology of Serbia, compared the effectiveness of digital PCR (dPCR) and quantitative PCR (qPCR) in detecting the *EGFR* T790M mutation in liquid biopsies of patients with disease progression on EGFR TKIs. Our findings reveal higher detection rate using dPCR (30.48%) compared to qPCR (19.44%). The improved sensitivity of dPCR supports its implementation as a preferred technique for *EGFR* T790M mutation detection, enhancing clinical decision-making and personalized treatment options for patients with NSCLC.

Introduction

Lung cancer is one of the leading causes of cancer-related deaths (18.7%) with the highest incidence worldwide (12.4% of all cancer cases) (1). In recent years, the incidence of lung cancer in Serbia has been steadily increasing, with a rate of 22.4 per 100,000 in women and 57 per 100,000 in men (2).

Molecular targeted therapies with the presence of corresponding alterations including the *EGFR*, *ALK*, *ROS1* and *KRAS* genes are already part of routine clinical practice. Screening for activating *EGFR*-TK (tyrosine-kinase) mutations in non-small cell lung cancer (NSCLC) is critical for several reasons, particularly for molecular profiling to tailor therapy to the patient, as well as to monitor therapy outcomes and determine prognosis. Certain *EGFR-TK* mutations and overall tumor heterogeneity may be the causes for the development of resistance to therapy (3).

Tyrosine-kinase inhibitors (TKI) are a type of targeted therapy designed to bind directly to the TK domain and inhibit further signaling. First-generation TKIs prevent ATP binding through their reversible interaction with the tyrosine kinase domain of *EGFR*, while second-generation inhibitors are characterized by their irreversible binding, which covalently bind to the TK domain (4). Osimertinib, a third-generation EGFR-TKI, was originally approved for the treatment of patients with EGFR T790M mutations after disease progression on first- or second-generation EGFR-TKIs and is now emerging as the standard first-line treatment for advanced *EGFR*-mutated NSCLC. This shift is supported by the results of the FLAURA trial, which showed a progression-free survival (PFS) of 18.9 months and an overall survival (OS) of 38.6 months(5). In Serbia, osimertinib was officially approved for first-line therapy in 2024. Therefore, it is important to improve the ability to monitor disease progression in order to select appropriate treatment options and strive for personalized patient care at each stage of disease evolution.

As repeating tissue biopsies in patients with progressive disease is often very difficult and uncomfortable for patients, liquid biopsies sometimes remain the only viable option for detecting mutations in the *EGFR* gene. Therefore, liquid biopsies allow the quantification of circulating cell-free DNA (cfDNA) to detect circulating tumor DNA (ctDNA), which contains specific tumor-associated genetic aberrations. However, the analysis of ctDNA poses several challenges and limitations due to its fragmentation as well as its low and variable concentration (6).

In Serbia, *EGFR* mutation testing from liquid biopsies for patients with tumor progression under EGFR-TKIs was introduced in 2016 and was performed on the Cobas qPCR instrument until 2023. To increase the sensitivity and specificity of mutation detection from liquid biopsy samples with a very low concentration of ctDNA, it was necessary to improve the technology. Therefore, dPCR technology was implemented in our laboratory from 2023 as a method that offers unparalleled sensitivity and precision in nucleic acid quantification and mutation detection. The technology is based on the application of optimal PCR conditions for the amplification of a single copy of the PCR template, dividing the sample into thousands of individual reactions, which allows precise quantification of the target sequences even when they are present at an extremely low level (low concentration of ctDNA). This partitioning, combined with the use of fluorescent probes, enables dPCR to detect and quantify rare mutations with much higher accuracy compared to conventional PCR techniques.

The aim of the study is to compare the detection rates of the T790M mutation in the *EGFR* gene in NSCLC patients at the Institute for Oncology and Radiology of Serbia over the past two years, using two different PCR platforms.

Patients and methods

Study cohort

This study included patients with lung adenocarcinoma (stage IIIb/IV, ECOG performance status 0, 1 or 2) who were diagnosed and treated at the Institute for Oncology and Radiology of Serbia over a two-year period. All patients had tumors with *EGFR* mutation, received first- or second-generation EGFR-TKIs in whom the tumor progressed and thus became candidates for genetic testing for the presence of resistant *EGFR* T790M mutation. The study included two cohorts of patients tested at the Department for Experimental Oncology, Laboratory for Molecular Genetics from March 2022 to February 2024. The first cohort was tested on the Cobas® platform from March 2022 to February 2023 and included 72 patients (39 females, 33 males, age range 65.8, median 67.5), while the second cohort was tested on Absolute Q™ DNA Digital PCR (dPCR) from March 2023 to February 2024 and included 76 patients (52 females, 24 males, age range 67.01, median 68).

DNA extraction

For quantitative PCR analysis, cfDNA was isolated from plasma, pleural effusion (PE) or cerebrospinal fluid using the Cobas® cfDNA Sample Preparation Kit (Roche Molecular Systems, Inc., Pleasanton, CA, USA). Circulating cell-free DNA for digital PCR was extracted from plasma and PE using the QIAamp® MinElute® ccfDNA Mini Kit (QIAGEN GmbH, Hilden, Germany). For cfDNA isolation QIAamp MinElute ccfDNA Mini Kit (QIAGEN, Hilden, Germany) isolation kit was used, which uses magnetic bead-based technology to enable the isolation of maximum amount of cfDNA. Concentration of cfDNA was measured using QubitTM dsDNA HS Assay Kit (Invitrogen Thermo Fisher Scientific, Waltham, United States). In both cfDNA extraction methods, extraction was performed by trained laboratory technicians in the Laboratory for Molecular Genetics at the Department of Experimental Oncology, following the manufacturer's instructions.

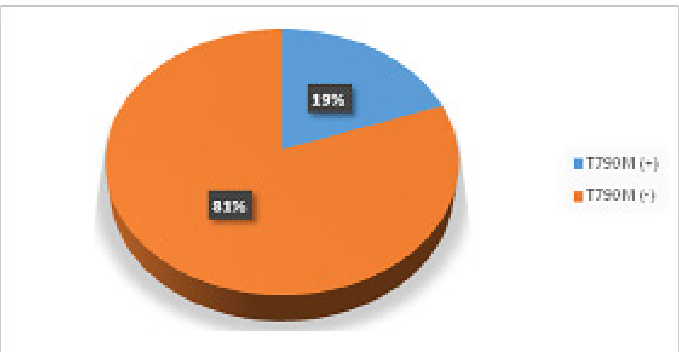

Figure 1. Percentage of T790M mutation detection using traditional qPCR technology on Cobas® platform

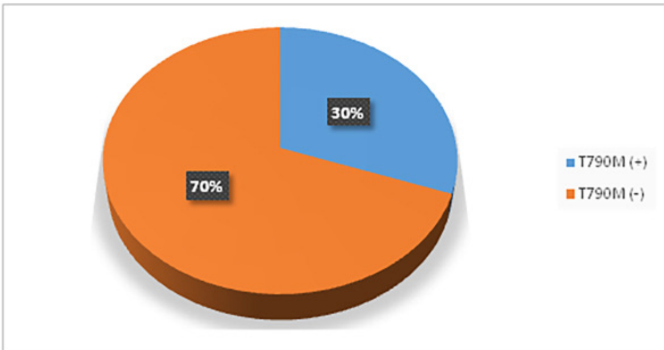

Figure 2. Percentage of T790M mutation detection using dPCR technology

PCR amplification and detection

The Cobas® *EGFR* Mutation Test v2 was utilized on the Cobas® 4800 platform. It is a test that provides mutation detection in exons 18-21 of the *EGFR* gene, including the T790M resistance mutation. *EGFR* dPCR mutation testing was performed using Absolute Q™ DNA Digital PCR MasterMix (5X), Taqman probe Hs000000029_rm for *EGFR* p. T790M and QuantStudio™ Absolute Q™ Isolation Buffer on QuantStudio™ Absolute Q™ MAP16 Digital PCR Kit 12-pack plates.

The detection was performed on Applied Biosystems QuantStudio Absolute Q Digital PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, SAD) and all samples were collected exclusively for the purpose of performing diagnostic procedures in order to adequately select the patients' therapy and to implement the analysis success statistics. The Laboratory for Molecular Genetics is annually certified by The European Molecular Genetics Quality Network. All analyses from this study are part of routine diagnostics procedures, approved by institutional Ethics Committee (approval no. 5665–01 from 17.12.2014.). All patients signed an informed consent.

Statistical analysis

Descriptive statistics were used to summarize the sample data. Fisher's exact test was used for statistical analysis. Two-sided p-values <0.05 were considered statistically significant.

Table 1. The number (percentage) of detected T790M mutations using qPCR and dPCR technology

	qPCR		dPCR	
	T790M (+)	T790M (-)	T790M (+)	T790M (-)
Total analyses performed	21 (19.44%)	87 (80.56%)	32 (30.48%)	72 (69.52%)
Plasma	17 (17.35%)	81 (82.65%)	23 (25.27%)	68 (74.73%)
Pleural effusion	4 (40%)	6 (60%)	8 (66.67%)	4 (33.33%)

Results

Across 108 analyses conducted on the Cobas® platform, 21 mutations were successfully detected, yielding a detection success rate of 19.44% (Figure 1). In some patients, detection was performed multiple times, bringing the total number of analyses to 72 patients. The detected mutations included 17 identified in plasma and 4 in PE. In some patients, the same mutation was found in both plasma and PE, resulting in 19 patients overall with detected mutations (Table 1). Among these 19 patients, 12 were females (63.16%) and 7 were males (36.84%). In 105 tests performed on the Applied Biosystems QuantStudio Absolute Q Digital PCR System, 32 mutations were identified, achieving a detection rate of 30.48% (Figure 2). The analyzes included a total of 76 patients.

Of these detected mutations, 23 were found in plasma samples, whereas 8 were found in PE. One female patient had the same mutation in both types of samples, leading to a total of 31 patients with detected mutations (Table 1). Out of these 31 patients, 19 were females and 12 were males, which implies 71.88% detected mutations in females and 37.5% in males.There was no significant difference between dPCR and qPCR *EGFR* T790M mutation detection rate (p=0.08, Fisher exact test). The result is not significant at p<0.05, but shows a trend towards statistical significance. The statistical significance potentially could be reached with more tested patients.

a.

b.

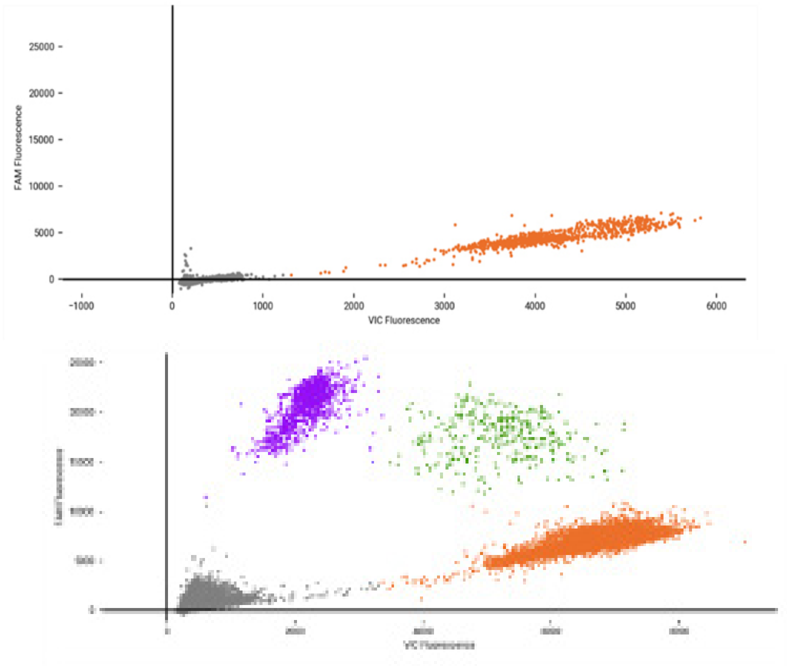

Figure 3. *EGFR* T790M mutation dPCR results: a.EGFR T790M mutation negative and b.EGFR T790M mutation positive result.

Concentrations of DNA extracted from the plasma/PE ranged 0,065-31.8 ng/µL (the highest DNA concentration which was extracted from plasma samples was 15.4 ng/µL). Examples of samples with and without present mutation are shown in Figures 3a and 3b, respectively.

Discussion

In non-squamous NSCLC there are numerous targetable mutations (*EGFR, ALK, ROS1, BRAF, KRAS, MET, HER2, RET, NRG1, NTRK1/2/3*...), therefore there are different testing strategies for the identification of driver mutations. The most common strategies are sequential or exclusionary single gene testing and NGS testing (7). Although currently the only model in Serbia is the sequential testing of single genes, several studies have shown that this approach is more expensive and less sensitive compared to NGS (7–9). In addition, it takes longer to obtain the results of the genetic test than with NGS analysis. The sensitivity of sequential testing of individual genes is lower, resulting in a lower number of patients being prescribed appropriate targeted therapy if the targeted mutations are present, which in turn leads to reduced OS (7,10).

Sequential testing of individual genes (*EGFR, ALK*) from tissue and liquid biopsy samples is currently being applied in Serbia. Taking into account the above-mentioned results, it is necessary to perform an analysis of the price evaluation of NGS tests compared to the prices of sequential tests, considering that fewer genes are currently approved for testing in Serbia than in the previously mentioned studies.

In Serbia, where the sequential approach was used at the time, patients were prescribed first- or second-generation EGFR-TKIs following the detection of a mutation in the *EGFR* gene. Although most patients initially respond to EGFR-TKI therapy, acquired resistance to EGFR-TKI develops in almost all patients after a progression-free period of 9 to 14 months, and a common cause of resistance is the occurrence of the T790M mutation in exon 20 of the *EGFR* gene (approximately 50% of cases) (11). When cancer progression is suspected, PCR testing for the T790M mutation has been recommended to determine whether third-generation EGFR-TKIs are indicated. Analysis of ctDNA in plasma and pleural effusion samples, a minimally invasive test, has been used as a valid and cost-effective alternative to tissue biopsies and identifies a large proportion of *EGFR* T790M mutations responsible for therapy resistance (12). If the mutation was not detected in patients in whom disease progression was suspected, some of them were advised to return for regular testing approximately every one to three months (patients with detected positive events on dPCR but below the established threshold) to repeat the PCR analysis, with the aim of subsequently attempting to detect the T790M mutation. If the resistance mutation is not detected, patients are referred to treatment with chemotherapy. Due to the low concentrations of ctDNA in the samples, the dPCR method was preferred because its higher sensitivity (13) increases the probability of detecting mutations even at minimal DNA concentrations (14,15). It is important to emphasize that in addition to the higher sensitivity of the dPCR method compared to qPCR, the use of different kits for the isolation of cfDNA, which work according to different principles, can also play an important role. There are several commercially available DNA isolation techniques, each utilizing different binding chemistries. These chemistries can impact both the efficiency and purity of DNA extraction, with each method having its own specific binding capacity. Common techniques include ethanol precipitation, anion-exchange resin (combined with ethanol precipitation), silica gel membrane binding, and magnetic silica particle binding. Among these, magnetic particle-based methods are more cost-effective, faster, and easier to scale and automate, while membrane binding methods tend to offer higher yields (16).

Comparing the percentage of dPCR analyzes that were positive for the T790M mutation from March 2023 to February 2024 (30.48%) with the percentage of positivity of tests performed on the Cobas platform from March 2022 to February 2023 (19.44%), greater success in detecting the desired mutation was observed with dPCR technology.

This study is significant, but has certain limitations. It is important because it shows that the more sensitive dPCR method achieves a higher detection rate of the resistant *EGFR* T790M mutation compared to the qPCR method, thus increasing the number of patients in whom third-generation EGFR-TKIs are indicated. On the other hand, the main limitation of this study is that the presence of this resistant mutation is only one of many mechanisms by which tumor resistance to therapy develops, and that some of the other mutations contributing to this resistance can only be detected by multiplex PCR for a larger number of mutations/genes or NGS sequencing.

For all the aforementioned reasons, the use of the most sensitive methods available for mutation detection is crucial. At the Institute for Oncology and Radiology of Serbia, dPCR technology is preferred, as it has shown higher sensitivity for the detection of point mutations compared to the qPCR Cobas platform. Although the dPCR method is more sensitive, it is not without limitations. Despite the fact that the cost per sample analysis is lower compared to qPCR, the main challenge/limitation is the lower possibility of multiplex PCR reactions. Given the fact that the focus of this method is currently on the detection of single mutations with higher sensitivity, the choice of this method is justified.

Conclusion

In comparison with conventional qPCR, which uses relative quantification, dPCR enables absolute quantification, offering significantly higher sensitivity and accuracy for detecting mutations. The study confirmed that dPCR technology demonstrated a higher detection rate of the T790M mutation over a one-year period, compared to the conventional qPCR method. Therefore, the detection of the T790M mutation in the *EGFR* gene in patients with NSCLC at the Institute for Oncology and Radiology of Serbia was significantly improved by the introduction of dPCR technology, which has led to enhanced diagnostic accuracy and efficiency in selecting appropriate therapies for patients with NSCLC.

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