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ORIGINAL ARTICLE

DROPLET DIGITAL PCR AS A MOLECULAR TOOL FOR THE DETECTION OF THE *EGFR* T790M MUTATION IN NSCLC PATIENTS WITH THE *EGFR* ACTIVATING MUTATIONS

Durgut S[1], Salihefendić L[1], Pećar D[1], Čeko I[1], Mulahuseinović N[1], Izmirlija M[3], Konjhodžić R[1,2]

[*]**Corresponding Author:** Selma Durgut, MD, ALEA Genetic Center, Olovska 67, 71000 Sarajevo, Bosnia and Herzegovina; Mob.: +38761904549, Email: selma.durgut@agc.ba

ABSTRACT

Background

Almost 50% of NSCLC patients who initially show a successful response to tyrosine kinase inhibitors targeted therapy (TKI therapy) eventually develop acquired *EGFR* T790M mutation. The T790M secondary mutation can cause resistance to the targeted therapy and disease relapse. Since this mutation can be present at very low frequencies in liquid biopsy samples, droplet digital PCR (ddPCR), due to its high sensitivity, has opened the possibility for minimally invasive monitoring of the disease during TKI targeted therapy.

Materials and methods

For this study, a total of 45 plasma samples from NSCLC patients with previously detected *EGFR*-activating mutations were analyzed. Extracted circulating free DNA was amplified and examined for the presence of T790M mutation using ddPCR technology. For the data analysis, QuantaSoft Software was used.

Results

Of 45 tested plasma samples, a total of 14 samples were identified as positive for the T790M mutation. The same samples eventually showed the presence of T790M mutation in FFPE. Droplet digital PCR showed its great advantage in high sensitivity detection of rare allele variants. Our ddPCR assay detected T790M mutant allele in frequencies from 0.1%. The average number of droplets generated by ddPCR was 9571.

Conclusion

Monitoring of the T790M mutation has an important role in the examination of the effects of the prescribed TKI therapy. Since monitoring of potential changes during TKI therapy requires repeated sampling, our results showed that ddPCR technology has made it possible to use liquid biopsy as an adequate minimally invasive alternative for single nucleotide polymorphisms (SNP) detection.

Keywords: ddPCR, liquid biopsy, NSCLC patients, T790M

INTRODUCTION

Lung cancer is one of the most commonly diagnosed cancers, while non-small cell lung cancers (NSCLC) account for 80-85% of all lung cancer cases[1,2]. Determining the presence of *EGFR* mutations in this type of lung cancer is an essential step, as it helps in selecting tyrosine kinase inhibitor therapy (TKI therapy). TKIs target the EGFR receptor in NSCLC patients and thus harbor activating *EGFR* mutations[3,4]. The presence of mutations in NSCLC is crucial for a patient's diagnosis, therapy assessment, and prognosis[5].

However, a significant number of patients who initially respond successfully to TKI therapy, eventually develop acquired resistance to EGFR-TKIs[6,7]. It has been found that specific secondary mutations occur and cause resistance to the applied therapy and lead to disease relapse[8,9]. The most frequent secondary mutation in NSCLC patients is a secondary mutation in exon 20, called the T790M mutation, which occurs in nearly 50% of cases where

[1] Alea Genetic Centre, Health Institute Alea Dr. Kandić, Sarajevo, Bosnia and Herzegovina

[2] Department of Laboratory Technology, Faculty of Health Studies, University of Sarajevo, Sarajevo, Bosnia and Herzegovina

[3] Faculty of Pharmacy, University of Sarajevo, Sarajevo, Bosnia and Herzegovina

the disease recurs in patients who have undergone TKI therapy[8]. As a result, third-generation EGFR-TKIs have been developed to target resistance mutation by blocking T790M mutant *EGFR* irreversibly[10].

Since secondary *EGFR* mutations, such as T790M, cause resistance to the prescribed TKI therapy, monitoring potential changes after the administration of therapy is crucial. This approach implies multiple sampling, and therefore the standard method of tissue biopsy, which is invasive, can make it difficult to resample regularly. To overcome this, liquid biopsy, which is minimally invasive, is becoming a more frequent method in clinical diagnostics[11]. It is also a suitable sampling method for patients in whom tumor formations have developed in inaccessible locations in the body or for those who may be at risk of some health complications in the event of a tissue biopsy[12].

Droplet digital PCR has several unique advantages, especially regarding rare mutation detection and precise DNA quantification[5]. Droplet digital PCR utilizes water-oil emulsion technology to partition each sample into 20 000 nanoliter-sized droplets, where target and background DNA are distributed randomly into the droplets. When properly designed, ddPCR can detect 1 mutant in 10 000 wild-type alleles[13]. Using ddPCR to test for mutation T790M from plasma samples, could be a promising approach for treating NSCLC patients.

MATERIALS AND METHODS

For this study, a total of 45 blood samples were collected from NSCLC patients who had previously tested positive for *EGFR*-activating mutations. The samples were collected at the Alea Genetic Center in Sarajevo. The selection of blood samples was based on identified *EGFR* mutations from lung adenocarcinoma tissue samples, and during this study they were tested for the presence of T790M mutation. The Alea Genetic Center did not perform the analysis for *EGFR*-activating mutations, and patients were selected according to their pathohistological and molecular testing results from different institutions. In this study, we concentrated solely on T790M mutation, activating *EGFR* mutations were determined by laboratories that tested the tissue samples.

Blood plasma was separated from the blood, with 1-4 ml taken from each sample and stored at a temperature of -20˚C. The circulating free DNA (cfDNA) was extracted from these plasma samples using QIAamp® Circulating Nucleic Acid Kit, following the manufacturer's instructions[14]. To process plasma samples faster and more efficiently, the QIAvac 24 Plus with the QIAvac Connecting System and QIAGEN Vacuum Pump was used according to the manufacturer's instructions[15]. Extracted cfDNA was quantified using Qubit 3 Fluorometer® and Qubit® dsDNA HS assay kit[16]. Starting DNA concentration for ddPCR reaction was 15 ng for every sample. Using Bio-Rad QX200 Droplet Digital PCR technology, all 45 samples were tested for a specific *EGFR* T790M mutation and its corresponding wild-type amplicons. The commercial assay used for T790M mutation and wild-type amplicon detection was PrimePCR™ ddPCR™ Mutation Detection Assay Kit: *EGFR* WT for p.T790M, and *EGFR* p.T790M. The multiplex assay was 20X concentrated and ddPCR supermix for probes was 2X concentrated. In order to portion each sample into droplets, they were placed into a QX200 droplet generator. Droplets were then transferred to a 96-well plate and placed into Bio-Rad T100 thermal cycler. The amplification was performed according to the manufacturer's instructions[17]. After DNA amplification, the droplet-containing plate was positioned in the QX200 droplet reader, allowing for the analysis of each droplet individually through a two-color system (FAM and HEX/VIC)[13]. For the analysis of the results, QuantaSoft Software was used.

When processing the results, wild-type and mutant T790M alleles were expressed as a number of FAM (mutant) and HEX (wild-type) droplets. The frequency of mutant alleles was determined by calculating the ratio of mutant droplets to the sum of mutant and wild-type droplets. Since ddPCR enables absolute quantification, mutant cfDNA templates were expressed as a number of copies per µl of tested DNA. The ddPCR threshold for positive mutant alleles was set at two or more positive droplets considering the added amount of DNA and generated number of droplets[18,19].

RESULTS

A total of 45 plasma samples were enrolled in this study. All selected plasma samples from NSCLC patients had previously shown the presence of *EGFR*-activating mutations on FFPE samples, in the following order: del19 (66.66%), L858R (17.77%), G719X (8.88%) and ins20 (4.44%). Out of 45 FFPE samples, one sample was invalidated for molecular testing for *EGFR*-activating mutations and only the patient's plasma sample was enrolled in the study.

Out of 45 samples, 14 samples were identified as positive for the T790M mutation, while 31 samples did not show the presence of the T790M mutant allele variant. The same 14 samples eventually showed the presence of T790M mutation in FFPE, while 31 tissue samples were identified as negative. In comparison to tumor tissue results, the sensitivity and specificity for *EGFR* T790M mutation in plasma samples were 100%. Our ddPCR as-

Table 1. Plasma samples that showed the presence of the T790M mutant allele variant

SAMPLE NUMBER	NUMBER OF DROPLETS	WILD TYPE/MUTANT T790M	T790M MUTANT ALLELE FREQUENCY (%)	MUTANT CONC (COPIES/ML)
3	6538	61/3	5	0.9
4	10038	7671/1634	12.3	209
6	8924	1534/8	0.5	0.8
8	12111	725/5	0.7	0.49
15	10891	480/4	0.8	0.43
18	8670	77/3	3.8	3
19	8015	210/7	3.3	1
20	12711	1428/2	0.1	0.28
34	7284	494/6	1.2	1
35	5775	577/25	4.1	5.1
36	14326	770/7	0.9	0.57
43	12788	632/16	2.5	1.5
44	12668	612/7	1.1	0.65
45	12789	536/18	3.3	1.7

say detected T790M mutant allele in frequencies ranging from 0.1%. The number of mutant molecules per µL of tested cfDNA ranged from 0.28 to 209 as depicted in Table 1. The average number of droplets generated by ddPCR was 9571.

As can be noticed from Table 1, plasma sample number 4 had an excessive amount of T790M mutant alleles. It is worth noting that the amount of tissue material from this patient was insufficient for valid results. Therefore, the result obtained from this tissue sample was reported as an invalid result and was not considered in the comparison between plasma and tissue samples. In the patient's plasma sample, 10 038 droplets were generated and the ratio of the number of droplets containing mutated gene variants to the total number of generated droplets was 12.3% (Figure 1).

However, in plasma sample 38, which was reported negative for the T790M mutation, one highly differentiated positive droplet was observed. For this sample, a small number of amplified copies were detected. To confirm the result, this plasma sample was run and analyzed twice, obtaining identical results, which indicates the accuracy of the procedure and the validity of the results. Although insufficient to be declared as positive, this result should not be ignored, and it indicates the need for liquid rebiopsy to determine the exact mutational status of the patient. This patient should be monitored for early detection of the T790M to adjust therapy if needed. Out of 14 plasma *EGFR* T790M positive samples, detected *EGFR*-activating mutations on FFPE samples were as follows: del19 (76.92%), L858R (15.38%), and G719X (7.69%).

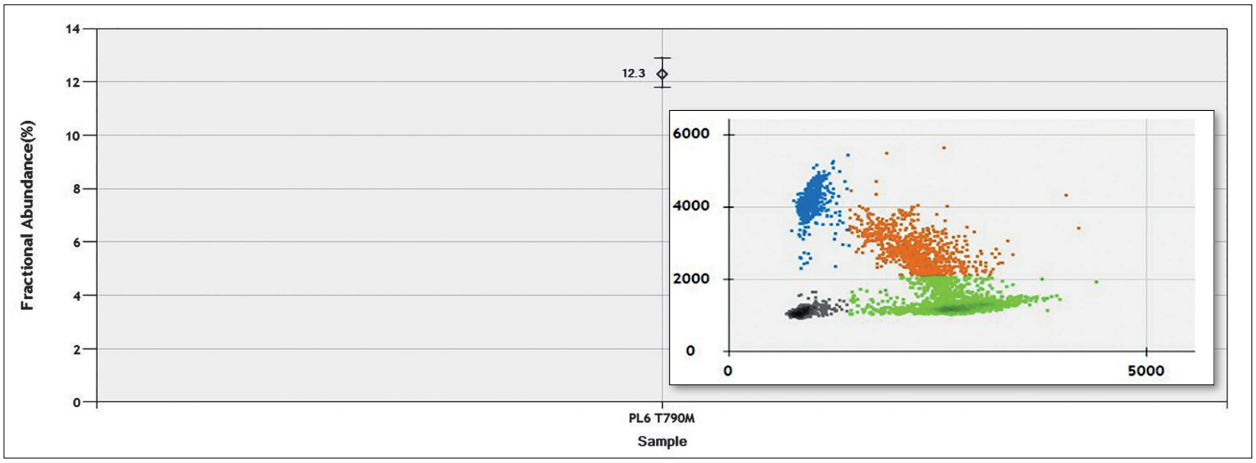

Figure 1. Sample number 4 with 12.3% mutant allele frequency

DISCUSSION

The main objective of this type of study is to improve the approach to treating oncology patients by providing individualized and accessible therapy. When one considers that around 50% of NSCLC patients experience disease relapse during TKI-targeted therapy and eventually acquire the T790M mutation, it becomes crucial to quickly discover molecular resistance mechanisms[20]. In current practice, the preferred method for monitoring resistance mechanisms is to perform a tissue re-biopsy. However, with the development of highly sensitive and reliable molecular technologies, such as ddPCR, analysis can be performed from other, minimally invasive types of samples[21].

Our study indicates that the sampling of patients can be significantly facilitated in order to monitor T790M status. Some studies estimate that up to 40% of relapsed NSCLC cases, cannot undergo molecular analysis due to issues with tumor tissue biopsies[22,23]. Two main factors contribute to this problem. Firstly, it is often challenging to obtain sufficient tissue material. As a result, clinicians use a well-known phrase "Tissue is an issue", since the patient's diagnosis is practically based on small amounts of tissue material. Secondly, tissue samples from NSCLC patients can be difficult to obtain since the lesions frequently develop in inaccessible locations[22]. For these patients, a more acceptable type of sample would be liquid biopsy, as an alternative to tissue re-biopsy. Although liquid biopsy generally contains a low concentration of circulating tumor biomarkers, highly sensitive and precise technologies such as ddPCR technology can overcome this drawback[25].

With its great sensitivity, ddPCR can accurately identify a mutant allele present at low frequency in a wild-type background. Since this technology has an advantage in mutation detection at low frequency, ddPCR has enabled more significant implementation of liquid biopsy in molecular diagnostics with the aim of early diagnosis of tumors, minimally invasive monitoring of disease, and therapy response assessment[25]. In this way, the sampling of patients is significantly facilitated and enables regular monitoring of the patient's healthcare condition.

In 2019, Salihefendić et al.[5] studied whether ddPCR technology could be used as a confirmatory method for the detection and quantification of somatic mutations, previously detected by NGS. A total of 35 samples from CRC and NSCLC patients were analyzed, and the results showed that there were no statistically significant differences between the results obtained by NGS and ddPCR methods. Therefore, high sensitivity and resolution of ddPCR, make it an adequate method for validating low-frequency somatic mutations[5].

In research published in 2016, Zheng et al.[27] reported that monitoring the status of cfDNA from plasma in NSCLC patients treated with TKI therapy can enable the detection of the acquired T790M mutation up to 6.8 months before the clinical progression of the patient's condition. In their research, almost half of the detected T790M positive patients were detected from plasma samples before disease progression (45.7%), and the time of detection varied from 0.8 to 6.8 months before clinical progression[27]. For patients with detected *EGFR* mutation who receive TKI therapy, regular analysis of the *EGFR* gene during treatment is significant in order to detect the T790M or other secondary mutations and to change the therapy.

CONCLUSION

Monitoring the T790M mutation has an important role in the examination of the effects of the prescribed TKI therapy. This approach implies multiple tissue biopsy sampling, which, as an invasive sampling method, has its limitations and risks. Our results showed that ddPCR technology has made it possible to use liquid biopsy as an adequate minimally invasive alternative for the detection of rare allele variants. Plasma ddPCR-based genotyping can be significant in the detection of specific mutations and patient monitoring. Using plasma as a sample for the detection of *EGFR* mutations by highly sensitive methods such as ddPCR can enable early detection of the T790M mutation, but also other inhibiting and activating mutations.

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CONFLICTS OF INTEREST:

There are no conflicts of interest.

REFERENCES

1. Garinet S, Laurent-Puig P, Blons H, Oudart J-B. Current and Future Molecular Testing in NSCLC, What Can We Expect from New Sequencing Technologies? J Clin Med. 2018; 7(6):144.
2. Rodak O, Peris-Diaz MD, Olbromski M, Podhorska-Okolow M, Dziegiel P. Current Landscape of Non-Small Cell Lung Cancer: Epidemiology, Histological Classification, Targeted Therapies, and Immunotherapy. Cancers. 2021; 13(18):4705.
3. Wu YL, Cheng Y, Zhou X, Lee KH, Nakagawa K, Niho S et al. Dacomitinib versus gefitinib as first-line treatment for patients with *EGFR*-mutation-positive

non-small-cell lung cancer (ARCHER 1050): a randomised, open-label, phase 3 trial. Lancet Oncol. 2017; 18:1454-66.

4. Soria J-C, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH et al. Osimertinib in Untreated *EGFR*-Mutated Advanced Non-Small-Cell Lung Cancer. N Engl J Med. 2018; 378(2):113-25.
5. Salihefendić L, Pećar D, Konjhodžić R. Validation of CRC and NSCLC somatic mutations detected with NGS using ddPCR. Genetics & Applications. 2019; 3(1):71-6.
6. Ohashi K, Maruvka YE, Michor F, Pao W. Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease. J Clin Oncol. 2013; 31:1070–80.
7. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with *EGFR*-mutant lung cancers. Clin Cancer Res. 2013; 19(8):2240-7.
8. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PloS Med. 2005; 2(3):73.
9. Cunha Santos G, Shepherd FA, Tsao MS. *EGFR* Mutations and Lung Cancer. Annual Review of Pathology: Mechanisms of Disease, 2011; 6(1):49–69.
10. Murtuza A, Bulbul A, Shen JP, Keshavarzian P, Woodward BD, Lopez-Diaz FJ et al. Novel third-generation EGFR tyrosine kinase inhibitors and strategies to overcome therapeutic resistance in lung cancer. Cancer Res. 2019; 79(4):689-98.
11. Rodriguez J, Avila J, Rolfo C, Ruiz-Patino A, Russo A, Ricaurte L et al. When Tissue is an Issue the Liquid Biopsy is Nonissue: A Review. Oncol Ther. 2021; 9(1):89-110.
12. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol. 2013; 10(8):472–484.
13. Droplet DigitalTM PCR Applications Guide (lab handbook). Hercules, California: Bio-Rad Laboratories Inc ; 2017.
14. QIAamp® Circulating Nucleic Acid Handbook. Hilden, Germany: Qiagen, 2019.
15. QIAvac 24 Plus Handbook (lab handbook). Hilden, Germany: Qiagen, 2010.
16. Qubit® dsDNA HS Assay Kits (lab handbook). Carlsbad, California: Life Technologies, 2015.
17. ddPCRTM *EGFR* T790M Screening Kit (lab handbook). Hercules, California: Bio-Rad Laboratories Inc ; 2016.
18. Kim Y, Shin S, Lee KA. Exosome-based detection of *EGFR* T790M in plasma and pleural fluid of prospectively enrolled non-small cell lung cancer patients after first-line tyrosine kinase inhibitor therapy. Cancer Cell Int. 2021; 21(50).
19. Ding PN, Becker T, Bray V, Chua W, Ma Y, Xu B, Lynch D, de Souza P, Roberts T. Plasma next generation sequencing and droplet digital PCR-based detection of epidermal growth factor receptor (*EGFR*) mutations in patients with advanced lung cancer treated with subsequent-line osimertinib. Thorac Cancer. 2019; 10(10):1879-1884.
20. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M et al. *EGFR* mutation and resistance of non-small-cell lung cancer to geftinib. N Engl J Med. 2005; 352(8): 786-92.
21. Wang W, Song Z, Zhang Y. A Comparison of ddPCR and ARMS for detecting *EGFR* T790M status in ctDNA from advanced NSCLC patients with acquired EGFR-TKI resistance. Cancer Medicine. 2016; 6(1): 154-162.
22. Yoon HJ, Lee HY, Lee KS, Choi, Y-L, Ahn M-J, Park K et al. Repeat biopsy for mutational analysis of non-small cell lung cancers resistant to previous chemotherapy: adequacy and complications. Radiology. 2012; 265(3): 939-948.
23. Chouaid C, Dujon C, Do P, Monnet I, Madroszyk A, Le Caer H et al. Feasibility and clinical impact of re-biopsy in advanced non small-cell lung cancer: a prospective multicenter study in a real-world setting (GFPC study 12-01). Lung Cancer. 2014; 86(2):170-3.
24. Silveira C, Sousa AC, Janeiro A, Malveiro S, Teixeira E, Brysch E et al. Detection and quantification of *EGFR* T790M mutation in liquid biopsies by droplet digital PCR. Transl Lung Cancer Res, 2021; 10(3): 1200-1208.
25. Jung A, Kirchner T. Liquid Biopsy in Tumor Genetic Diagnosis. Deutsches Arzteblatt international, 2018; 115(10): 169–174.
26. Chan KCA, Jiang PY, Zheng YWL, Liao GJW, Sun H, Wong J et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem, 2013; 59(1): 211–24.
27. Zheng D, Ye X, Zhang MZ, Sun Y, Wang JY, Ni J et al. Plasma *EGFR* T790M ctDNA status is associated with clinical outcome in advanced NSCLC patients with acquired EGFR-TKI resistance. Scientific Reports, 2016; 6,20913.