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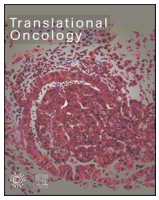

EGFR mutation testing from pleural effusions of non-small cell lung cancer patients at the institute for oncology and radiology of Serbia

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ABSTRACT

Background: The use tumor-derived cell-free DNA extracted from body fluids is being evaluated for genetic testing in lung cancer. The aim of this study was to explore the feasibility and utility of implementation of *EGFR* molecular testing from pleural effusions in non-small cell lung cancer in the clinical diagnostics workflow.
Patients and methods: This study included patients diagnosed with primary lung adenocarcinoma in the period July 2016 to June 2023. *EGFR* mutation testing was performed by qPCR (Cobas®) and dPCR. Testing was performed from 211 plasma samples when tissue was unavailable at diagnosis, and from 301 plasma samples and 18 pleural effusions at progression on first/second generation of EGFR TKIs. Descriptive methods of statistical analysis were used to summarize the sample data. Fisher's exact test, McNemar's test, Cohen's kappa tests were used for statistical analyses. Two-sided p-values <0.05 were considered statistically significant.
Results: A significantly higher detection rate of the T790M mutation in pleural effusion was obtained compared to blood (50% and 20%, $p=0.047$). When comparing the detection success rate of the resistant T790M mutation in pleural effusion and blood, a statistically significant difference was obtained in favor of pleural effusion (50% vs. 21.87%, $p=0.01$).
Conclusions: Superior performance of pleural effusions compared to blood plasma was shown both in the analysis of success rate and in the detection of the resistant T790M mutation, at progression on EGFR TKIs. Pleural effusion should be considered in this setting whenever available, especially in countries with limited health resources.

Introduction

Molecular targeted therapies matched with driver mutations, such as epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), and ROS proto-oncogene 1 (*ROS1*) in patients with advanced stage non-squamous non-small cell lung cancer (NSCLC), are already the standard in everyday clinical practice, largely surpassing conventional chemotherapy in improving patient survival [1]. This ever-expanding number of targeted therapies imposes a need for predictive molecular genetic testing as a first step to select the patients for a tailored therapeutic approach with maximum efficacy. However, in daily practice it is not always possible to obtain sufficient tumor tissue for genetic testing. Access to lung tissue samples is restricted and tumor biopsy often ends with samples of insufficient quality or quantity. Thus, the use of tumor-derived cell-free DNA (cfDNA) extracted from body fluids, including plasma, pleural effusions (PE), cerebrospinal fluids, urine, and saliva, are being investigated in order to evaluate their suitability for genetic testing [2,3]. Liquid biopsies are minimally invasive and potentially superior at representing intra- and inter-tumor heterogeneity than single tumor biopsies.

Lately, the restrictions of blood-based cfDNA approach prompted the use of other accessible liquid resources, such as urine, cerebrospinal fluid and in lung cancer, particularly, pleural effusion [4]. Malignant pleural effusion represents a common complication of lung cancer, defined as excessed fluid accumulated between lung and pleural cavity. PE is most frequently a complication of progressive lung carcinomas, which affects from 7 to 23% of the patients, possibly could reach up to 40% of the patients during the course of the disease [5,6] and is often related with the resistance to chemo- and targeted therapies [7]. After assessing the pleural fluid, the sample is processed by centrifugation, whereby two fractions are obtained. The first fraction is cell sediment, used for cytology, and the second is cell-free supernatant, mainly used

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for biochemical analyses. The biggest challenge is the clarification of the neoplastic origin of the pleural effusion, which is achieved by cyto-morphological analyses that have sufficient sensitivity to identify the exact cellular composition and prove the presence of malignant cells [8,9]. Effusion derived tumor cells enriched for testing can be the subject for phenotypic studies (flow cytometry), and furthermore, cell-block processing of the sediment could provide the tissue for the analysis, similar to formaldehyde-fixed and paraffin-embedded (FFPE) sample [10]. Cell samples have been shown to be a good source of DNA for molecular diagnostic analyzes in lung cancer, such as determining mutations in the *EGFR, KRAS* or *BRAF* genes. However, cell sediment testing had an average sensitivity of around 60% (from 40 to 87%), revealing a distinct limitation of this approach [11]. cfDNA in the supernatant of PE has been proved to be superior to cell sediment in detection of genetic variants. Fragment size of cfDNA is diverse in different types of body fluids. In plasma, cfDNA fragments have an average size of 160 bp. In comparison with plasma, cfDNA in supernatant of PE contains significantly longer DNA fragments varying from 300–500 bp [12,13]. It remains to be verified in laboratory practice whether these longer fragments increase the sensitivity of genetic testing from PE.

Earlier studies clearly demonstrated the utility of PE for *EGFR* mutation testing by PCR in lung adenocarcinoma patients [14,15]. Even so, serial single-gene testing could use up the remaining material and prove insufficient for precision medicine, when multiple biomarkers need to be analyzed, as in lung cancer. Next generation sequencing (NGS) can simultaneously detect and quantify, in a massive parallel and high throughput manner, multiple genomic alterations such as point mutations, insertions, deletions, gene fusions, and amplifications in multiple specimens. NGS only needs a single DNA or RNA input from each sample, sparing precious material that has high analytical sensitivity, while it provides comprehensive molecular coverage and is cheaper per base compared to sequential single biomarker testing [16,17]. Although the efficacy of NGS for molecular profiling from PE cfDNA was demonstrated, large-scale studies are needed to prove clinical relevance of this approach. In addition to next-generation sequencing (NGS), digital PCR offers a promising avenue for effectively tracking particular resistance mutations like T790M or others. Few studies compared digital PCR with other traditional PCR methods and their results demonstrated high diagnostic accuracy of this method [18,19]. Digital PCR is known for its exceptional sensitivity, speed, and cost-effectiveness, holds significant potential for practical implementation in clinical settings, especially for overseeing specific mutations.

The majority of the published studies thus far have primarily focused on investigating the detection of clinically significant mutations in pleural effusion samples. However, the translation of these research findings into the practical clinical setting for the purpose of selecting standard-of-care therapies is accompanied by distinct challenges. In our study, we aim to address this gap by presenting the implementation of pleural effusion testing within the clinical diagnostic workflow of a prominent cancer center. Through this research, we aim to bridge the gap between scientific knowledge and real-life patient care, providing valuable insights into the integration of pleural effusion testing as a routine component of clinical practice, especially in LMIC countries with limited health resources.

Methods

Patient samples

This study included patients with primary lung adenocarcinoma (stage IIIb/IV, ECOG performance status 0, 1 or 2) of Caucasian descent in the period from July 2016 to June 2023. The presence of *EGFR* mutations was tested from plasma samples in 211 cases when tissue samples were unavailable at diagnosis and from plasma samples in 301 cases after progression on first/second generation of *EGFR* TKIs. The sample size met the criteria of a minimum number of samples taking into account the incidence of *EGFR*-mutated lung cancer presenting with pleural effusion and population size in Serbia (95% confidence level). Patients with sensitizing *EGFR* mutations received first or second-generation *EGFR* TKIs (gefitinib, erlotinib, afatinib) until progression or appearance of unacceptable toxicity. Furthermore, *EGFR* testing was performed from PE in 18 cases after progression on first/second generation of *EGFR* TKIs.

EGFR mutation testing

Circulating free DNA was extracted from plasma or PE using the Cobas® cfDNA Sample Preparation Kit and QIAamp® MinElute® ccfDNA Mini Kit. *EGFR* mutation testing was performed using the Cobas® *EGFR* Mutation Test v2 on Cobas® 4800 (Roche Diagnostics) and TaqMan probes Hs000000029_rm and Hs000000026_rm for *EGFR* p. T790M and p. L858R mutations, respectively, and Absolute Q™ DNA Digital PCR Master Mix (5X) on Applied Biosystems QuantStudio Absolute Q Digital PCR System (Thermo Fisher Scientific), according to manufacturers' protocol (catalog number A52864; publication number MAN0025621). All analyses were performed in the Laboratory for Molecular Genetics until end of June 2023. The methodology is based on prior published work by our research group [20,21]. 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.). This is a research academic study, and all patients signed an informed consent.

Statistical analysis

Descriptive methods of statistical analysis (frequencies, percentage, median) were used to summarize the sample data. Fisher's exact test, McNemar's test, Cohen's kappa tests were used for statistical analyses of associations between *EGFR* mutation status/type and patient's characteristic (gender, age). Two-sided p-values less than 0.05 were considered statistically significant. The statistical analyses were performed using GraphPad Prism (V.8.0.1 GraphPad Software, CA, USA), and online SciStatCalc (https://www.scistat.com/statisticaltests/) and GraphPad by Dotmatics calculators.

Results

Patients' characteristics

Patients' characteristics are shown in Table 1. The group of patients tested from liquid biopsy (blood) at baseline consisted of 117 males (55.45%) and 94 females (44.55%), with an age range of 31–86, and a median of 66 years. Two groups of patients from liquid biopsies (blood and PE) were also tested at progression on first/second generation *EGFR* TKIs and consisted of 111 males (36.88%) and 190 females (63.12%) for plasma, and 4 males (22.22%) and 14 females (77.78%) for pleural

Table 1
Patients' characteristics by sample type and status.

Characteristic	Baseline testing	Testing at progression on *EGFR* TKIs	
	Liquid biopsy (blood) (*n*=211) n (%)	Liquid biopsy (blood) (*n*=301) n (%)	Liquid biopsy (pleural effusion) (*n*=18*) n (%)
Sex			
Male	117 (55.45)	111 (36.88)	4 (22.22)
Female	94 (44.55)	190 (63.12)	14 (77.78)
Age, years			
Range	31–86	34–91	43–79
Median	66	67	64

* Patients who were also tested from blood

effusion, with an age range of 34–91 (median 67), and 43–79 (median 64) years, respectively. The samples of the tested groups were received from various health centers in Serbia as a part of centralized *EGFR* testing.

Baseline EGFR mutation testing

The results of *EGFR* testing are shown in Table 2. Testing of liquid biopsy samples at baseline was successful in 211/217 (97.24%) samples when tissue was not available for testing. Twenty-two mutated samples (10.14%) were detected with a turnaround time of 2 working days. There was a statistically significant difference to provide a clinically informative result in success rates between FFPE (success rate of 99.32% previously determined for external accreditation purposes) and blood plasma, in favor of liquid biopsy (p=0.006, Fisher's exact test). The success rates reflected on the clinical implementation of the test to give informative results, rather than the head-to-head comparison of the analytical sensitivity.

The frequencies of *EGFR* mutations detected from blood plasma were 63.64% for ex19del, 13.64% for L858R, 4.55% for L861Q, 4.55% for insertions in exon 20, 4.55% for S768I and 4.55% for double mutants. The T790M resistant mutation was detected in only 1 sample (4.55%). Mutations were detected more often in female than in male patients, although statistical significance was not reached. No statistically significant difference was observed in the detection rates of *EGFR* mutations between FFPE tissue (9.7% for FFPE, previously determined for external accreditation purposes) and blood samples at baseline.

EGFR mutation testing at progression on EGFR TKIs

Patients who progressed on 1st and 2nd generation *EGFR* TKIs were tested for the presence of *EGFR* mutations from liquid biopsy. A total of 301 patients were tested. Some patients were re-tested multiple times in one-month periods in an effort to detect the resistant T790M mutation using repeated liquid biopsies, which amounted to a total of 407 analyses. The analysis success rate was 99.75%. Mutations in the *EGFR* gene were detected more often in females than in males (63.57% vs. 52.70%, respectively), with a statistical significance of p=0.036 (Fig. 1B). Further stratification by mutation type showed a difference in the occurrence of most common ex19del and/or L858R mutations in females and males (63.69% vs. 41.33%, respectively), with a statistical significance of p=0.002.

Difference in *EGFR* mutational status in blood samples at progression compared to median age was observed but did not reach statistical significance (Fig. 1E). Some patients were tested at baseline in another testing center, so we did not have data on the type of primary mutation detected from FFPE tissue for all patients who progressed on 1st and 2nd generation of *EGFR*-TKIs. For 142 patients whose data was available, matching with the primary mutation was obtained in 93 patients (65.5%), while the primary mutation detected in FFPE tissue samples was not confirmed in blood samples of 49 patients (34.5%). The results showed that testing the presence of *EGFR* mutations in blood at progression did not match the primary mutations detected from FFPE tissue samples with a high statistical significance of $p<0.0001$ (McNemar's test with Yate's correction 0.5, $p<0.0001$) (Table 4).

Liquid biopsies (blood) samples from patients who were tested more than once were classified as "rebiopsies" in order to monitor the success of the detection of the resistant T790M mutation. Our results showed that the detection rate of the primary mutation was lower in the first testing from the liquid biopsy compared to the rebiopsy, while the situation with the detection of the primary T790M mutation was reversed (Fig. 2A, $p<0.0001$). The total detection percentage of the resistant T790M mutation from liquid biopsy samples (blood plasma) was 36.77% in the mutated group. Detection of primary mutation (ex19del, L858R) in paired liquid biopsy samples at progression on *EGFR* TKIs (blood and pleural effusion) is presented in Table 5. Analysis between these two types of samples was examined by Cohen's kappa test, and the obtained result kappa=0.545 indicates a moderate agreement of the detection of primary mutation.

In the last 6 years, testing from PE of patients who progressed on first/second generation *EGFR* TKIs was employed with the aim of increasing the detection rate of the resistant T790M mutation. Twenty analyses were performed from blood and PE of 18 patients concurrently. The obtained results indicated a significantly higher detection rate of the T790M mutation in PE compared to blood (50% and 20%, respectively, p=0.047, two-sample proportion test). The agreement in the detection of the resistant T790M mutation between these two types of samples was examined by Cohen's kappa test, and the obtained result kappa=0.399 indicates a fair agreement of the detection of this mutation (Table 6).

When comparing the detection success rate of the resistant T790M mutation in blood and PE, a statistically significant difference was obtained in favor of PE (21.87% and 50%, respectively, p=0.01, Fig. 2B). Distribution of detected *EGFR* mutation types in PE samples at progression is presented in Fig. 3, depicting similarity in the detection mutation profile with blood.

From March 2023, we employed dPCR for *EGFR* mutation testing from liquid biopsy samples (blood and pleural effusion) for patients who progressed on 1st and 2nd generation of *EGFR*-TKIs. Until June 2023, 30 liquid biopsy samples (25 blood and 5 PE samples) were analyzed, both on qPCR and dPCR in parallel. The results are presented in Table 3. In case of qPCR *EGFR* mutation testing, we observed 6/30 (20%) T790M positive samples (4/25 T790M positive blood samples (16%) and 2/5 T790M positive PE samples (40%)). In case of dPCR *EGFR* mutation testing, we observed 10/30 (33.33%) T790M positive samples (6/25 T790M positive blood samples (24%) and 4/5 T790M positive PE samples (80%)). Agreement between these two types of analysis was examined by Cohen's kappa test, and the obtained result kappa=0.545 indicates a good agreement of the detection of T790M mutation (Table 7).

Table 2
Distribution of *EGFR* mutation types in patient liquid biopsy samples.

	Baseline testing	Testing at progression on *EGFR* TKIs	
	Liquid biopsy (blood) (n=217) n (%)	Liquid biopsy with rebiopsy (blood) (n=407) n (%)	Liquid biopsy (pleural effusion) (n=20) n (%)
EGFR status			
EGFR mut	22 (10.14)	242 (59.46)	16 (80)
EGFR wt	189 (87.10)	164 (40.29)	4 (20)
NA	6 (2.76)	1 (0.25)	0 (0)
EGFR mutation type			
Ex19del	14 (63.64)	92 (38.02)	5 (31.25)
L858R	3 (13.64)	36 (14.88)	1 (6.25)
L861Q	1 (4.55)	3 (1.24)	0 (0)
G719X	0 (0)	5 (2.07)	0 (0)
Ex20Ins	1 (4.55)	14 (5.78)	0 (0)
S768I	1 (4.55)	0 (0)	0 (0)
Double mutants	1 (4.55)	91 (37.60)	10 (62.50)
Triple mutants	0 (0)	1 (0.41)	0 (0)
T790M	1 (4.55)	89** (36.77)	10*** (62.50)

*Concomitant with L858R mutation;
** concomitant with ex19del in 65 samples, with L858R in 23 samples, with G719X in 1 sample, with a double mutant (G719X+S768I) in 1 sample;
*** concomitant with ex19del in 7 samples and with L858R in 3 samples.

Discussion

Serbia has one of the highest age-standardized incidence (22.4 per 100,000 person-years) and mortality rates (93.4 for men, 40.7 for women, per 100,000 person-years) of lung cancer worldwide [22], with

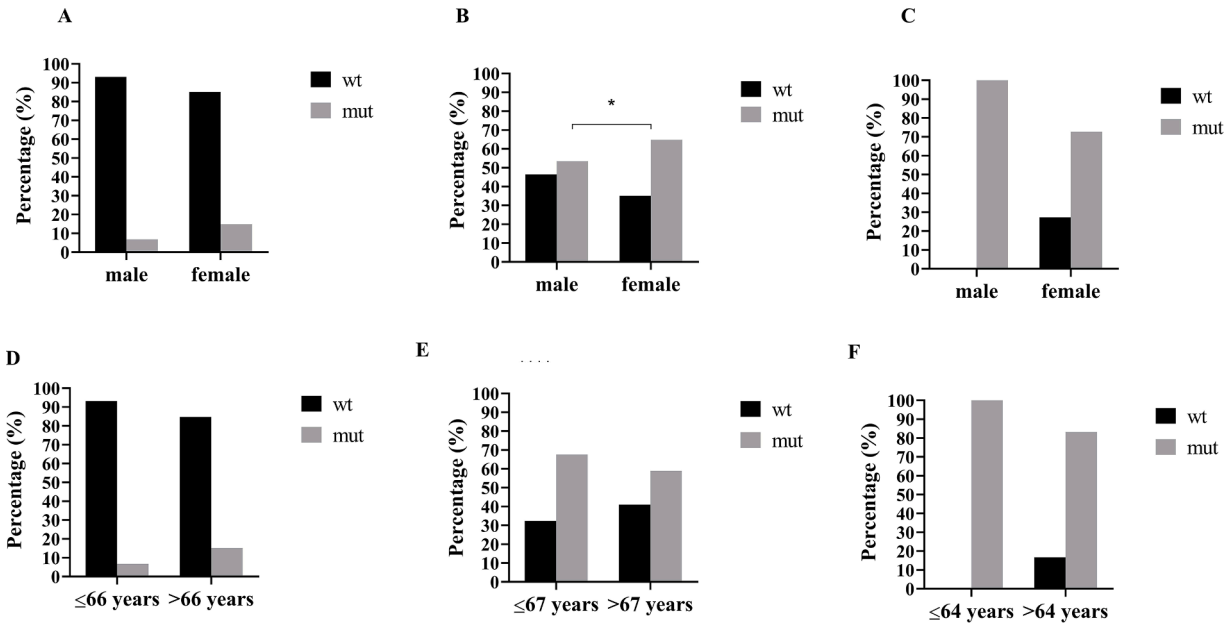

Fig. 1. Percentage of detected *EGFR* mutations according to sex of patients at diagnosis in plasma samples **(A)** and at progression on *EGFR* TKIs in liquid biopsy (blood and pleural effusion) samples **(B,C).** Percentage of mutated samples according to median age at diagnosis in blood samples **(D)** and in blood and pleural effusion samples at progression **(E,F)**. *p=0.036.

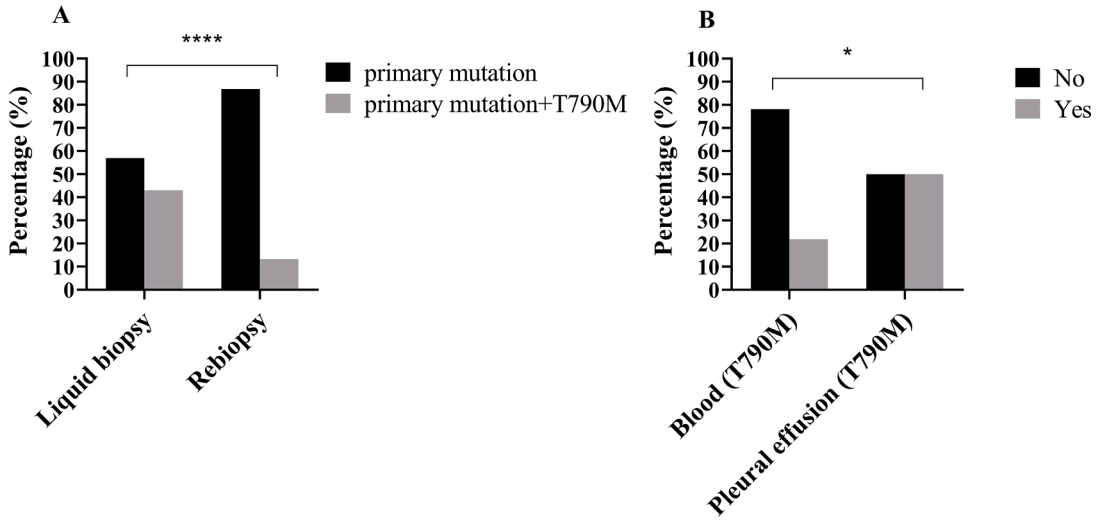

Fig. 2. *EGFR* mutation detection of primary and T790M mutation at progression on *EGFR* TKIs: in liquid biopsy and rebiopsy blood plasma samples **(A)** and in blood plasma overall and pleural effusion samples **(B)**. *p=0.01, ****p<0.0001.

adenocarcinoma being the most common subtype. High prevalence of smoking, air pollution and the lack of national lung cancer screening program might be considered as crucial factors for this, as most lung cancer patients are diagnosed in advanced stage of disease. The detection of lung cancer in early stages has been especially low in the last two years, during the COVID-19 pandemic [23]. Anticipating the rise of newly diagnosed cases in advanced stages of the disease, better management strategies for these patients are needed. Approaches that have evaluated population specific risk factors, and prognostic and predictive biomarkers in advanced disease stages have been explored in the past in our country in an effort to reduce the national incidence and mortality rates [24–29]. In 2008, a centralized pharmacogenetics service was established at the Department of Experimental Oncology, performing molecular testing for companion diagnostics as a referral center for the territory of central and southern Serbia covering a population of over 4 million [21]. The majority of tests performed within the pharmacogenetics diagnostics workflow are based on FFPE tissue, with very high success rates (99.9%) and rapid turnaround time (5–7 days).

Minimally invasive approaches based on the use of liquid biopsy such as blood plasma or pleural effusions have a great potential for the detection of actionable *EGFR* mutations both at diagnosis when tissue samples are scarce or not available, and for resistance monitoring during *EGFR*-TKI treatment. cfDNA is increasingly used in the clinical oncology setting, with the first EMEA- and FDA- approved IVD test for *EGFR* T790M mutation detection indicated as companion diagnostics creating an opportunity for third generation TKI therapy. The analysis of cfDNA has great value as a diagnostic analyte when repeated biopsies are not possible or feasible. In metastatic patients, cfDNA enables detection of mutations originating from both primary tumor and distant metastasis in an unbiased manner. Testing for *EGFR* mutations has been indicated at diagnosis or progression as companion diagnostics for *EGFR* TKI therapy worldwide [30,31]. Testing for T970M point mutation after

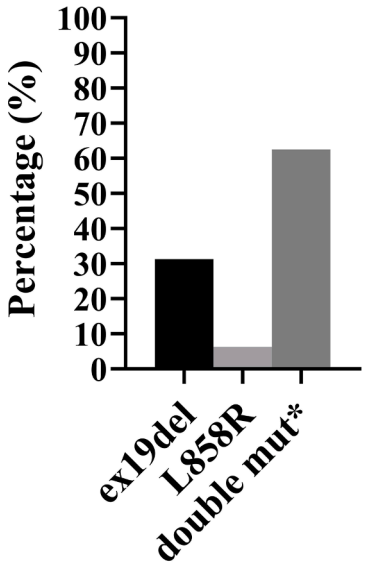

Fig. 3. Distribution of detected *EGFR* mutation types in PE samples at progression.

Table 3
Distribution of *EGFR* mutation types in patients' liquid biopsy samples at progression on *EGFR* TKIs (analyzes were done by qPCR and dPCR).

	Cobas® 4800		QuantStudio Absolute Q Digital PCR	
	Liquid biopsy with rebiopsy (blood) (*n*=25) n (%)	Liquid biopsy (pleural effusion) (*n*=5) n (%)	Liquid biopsy with rebiopsy (blood) (*n*=25) n (%)	Liquid biopsy (pleural effusion) (*n*=5) n (%)
EGFR status*				
EGFR mut	11 (44)	3 (60)	7 (28)	4 (80)
EGFR wt	14 (56)	2 (40)	18 (72)	1 (20)
NA	0 (0)	0 (0)	0 (0)	0 (0)
EGFR mut type				
Ex19del	6 (54.54)	1 (33.33)	/	/
L858R	1 (9.10)	0 (0)	1 (14.29)	0 (0)
Double mut**	4 (36.36)	2 (66.67)	2 (28.57)	1 (25)
T790M***	4 (36.36)	2 (66.67)	4 (57.14)	3 (75)

* In case of dPCR EGFR mutation detection assays for L858R and T790M were used only;
** T790M concomitant with Ex19del or L858R;
*** T790M only in case of dPCR mutation testing and concomitant with Ex19del or L858R in case of qPCR mutation testing.

Table 4
Detection of the same primary mutation in paired FFPE tissue and liquid biopsy samples (blood and pleural effusion) at progression on *EGFR* TKIs.

FFPE at diagnosis	Liquid biopsy (blood at progression)	
	Yes	No
Yes	93	49
No	0	0

*McNemar test with Yate's correction of 0.5; $p<0.0001$.

progression while on first/second generation *EGFR* TKI has been recommended by clinical guidelines since 2013 [31,32]. In Serbia, *EGFR* mutation testing from liquid biopsy of patients who have progressed on *EGFR* TKIs was introduced in 2016 [20].

Here we present real-world results from a pilot study initiated in 2021 to evaluate feasibility and utility of implementation of *EGFR* molecular testing from PE for lung cancer pharmacogenomics, in a low-and middle-income country (LMIC) setting. To the best of our knowledge, this is a first study to report real-world data on *EGFR* mutation testing comparing success rates, detection rates and distribution of mutation types for blood plasma and PE in a LMIC setting. Previous work with a small number of pleural effusion samples focusing on a wider range of genes using ddPCR (droplet digital PCR) and NGS (next-generation sequencing) demonstrated feasibility of using PE for mutation testing and motivated our own research [33,34].

Presently, the Department of Experimental Oncology of the Institute for Oncology and Radiology of Serbia (IORS) employs the real-time PCR method to conduct mutation testing in the *EGFR* gene, while the integration of dPCR for diagnostic purposes has begun in March 2023. To the best of our knowledge, these are the first literature results of *EGFR* testing in lung cancer using this methodological approach. At this time, the implementation of NGS for upfront genetic testing of patients with advanced NSCLC would not yield significant clinical benefits in LIMC primarily due to the considerable cost per sample for testing and the time-consuming nature of the process and a small number of targeted therapies covered by health insurance funds.

Adequacy of a sample for mutation testing at diagnosis depends on several technical and biological factors, including mode of sample preservation, tumor heterogeneity, tumor-derived DNA content and its quantity and quality. Success rates at progression for blood plasma and pleural effusion samples were 99.75% and 100%, possibly reflecting the higher cfDNA quantity and ctDNA content compared to baseline.

Prevalence of *EGFR* mutation at diagnosis in lung adenocarcinoma varies between different populations, yet there is little data on *EGFR* mutation prevalence among Slavic patients. In our cohort of liquid biopsy samples of south-Slavic patients we observed a lower prevalence at diagnosis (10.14%) compared to those reported in Caucasian western-European [35] (15%), Russian [36] (18%) and Asian [37] (51%) patients with NSCLC. The lower *EGFR* mutation prevalence in Serbia might

Table 5
Detection of primary mutation (ex19del, L858R) in paired liquid biopsy samples at progression on *EGFR* TKIs (blood and pleural effusion).

Blood	Pleural effusion	
	Yes	No
Yes	12	0
No	4	4

* Cohen's kappa test; kappa=0.545 – "Moderate agreement".

Table 6
Detection of the resistant T790M mutation in paired liquid biopsy samples at progression on *EGFR* TKIs (blood and pleural effusion).

Blood	Pleural effusion	
	Yes	No
Yes	4	0
No	6	10

* Cohen's kappa test; kappa=0.399 – "Fair agreement".

Table 7
Detection of the resistant T790M mutation in paired liquid biopsy samples at progression on *EGFR* TKIs (blood and pleural effusion) on qPCR and dPCR.

QuantStudio Absolute Q Digital PCR (T790M)	Cobas® 4800 (T790M)	
	Yes	No
Yes	6	4
No	0	20

* Cohen's kappa test; kappa=0.667 – "Good agreement".

reflect the high-burden of epidemiological environmental factors such as smoking rates and air-pollution influencing lung carcinogenesis. Approximately one-third of adult population in Serbia are smokers, while the content of PM2.5 and PM10 atmospheric particles in urban centers are among the highest in the world in the past decade [38]. Thus, lung cancer screening for individuals who never smoked [39] might also be considered of high importance in order to reduce the number of patients diagnosed in advanced stages for which *EGFR* TKI therapy is approved in our country.

Selective pressure of *EGFR* TKI therapy directs tumor evolution towards acquisition of resistance phenotypes. One such mechanism is mediated through secondary *EGFR* T790M mutation. Repeated biopsy and surgical excision are not always possible and carry additional costs and risks of complications. Implementation of non-standard analytes in the molecular diagnostics workflow, such as blood plasma or PE and aspirates provide a valuable sample source of tumor-derived cfDNA allowing detection of somatic mutations. In a liquid biopsy sample at progression, we expect to observe both the primary and secondary mutation, however we noted a high discordance in mutation type for matched patients between tissue and blood plasma, in line with previous reports [40], indicating false negative calls. However, in case of heterogenic tumors, liquid biopsy may be in favor for some patients. It has also been suggested that liquid biopsy testing might be more cost-effective and less invasive to be performed prior to tissue evaluation for diagnostic purposes for a larger subset of patients, due to faster turnaround time and lack of false positivity [41]. In a subset of cases, a resistance mutation was detected in blood plasma at progression, but not the primary one highlighting the technical limitations of a liquid biopsy-based test. The possible cause for this discrepancy could be the sampling error due to low cfDNA concentrations in plasma and procedural loss, or the highly fragmented nature of cfDNA preventing PCR amplification. More sensitive methods based on ddPCR using shorter amplicons might increase the sensitivity and concordance rates, as was observed from our first results using this method.

At IORS, the current clinical workflow for *EGFR* mutation testing in patients with disease progression involves initially confirming disease progression through clinical assessments such as CT scan or medical examinations. Based on the successful application of PE for mutation detection and previous work demonstrating earlier detection of molecular relapse vs. clinically detectable relapse by imaging methods, it would be worth pursuing further applications for disease monitoring during systemic therapy. This stems from the ability to detect molecular disease progression earlier by performing genetic testing on liquid biopsies to identify the presence of the resistant T790M mutation [33,42].

In a subset of our patients tested concurrently using blood plasma and PE there was a significant difference in detection rates of T790M mutation in favor of PE, likely stemming from an enrichment of cfDNA in these samples compared to plasma. This is in accordance with the literature data, where the concentrations of circulating DNA, as well as the presence of DNA fragments of longer length are significantly more represented in the samples of pleural effusion in relation to blood plasma samples [12,43]. PE fluid specimens had recently also shown the highest adequacy and detection rate when comparing multiple cytology specimen types [44]. PE is often removed to relieve symptoms, so material is already available for a subset of patients (with dyspnea). Moreover, other targetable mutations can be detected in PE. Although it would be useful for all patients in whom PE is detected to undergo pleural puncture, this is not a routine clinical practice at IORS or other hospitals that refer their patients' samples to our laboratory. Firstly, the isolation/puncture of PE is not performed in patients having a smaller amount of PE if there are no other complications (comorbidities). Furthermore, the medical centers sending pleural effusion samples to our laboratory adhere to clinical guidelines for *EGFR* mutation testing only in case of cytologically positive PE, which is debatable, regarding literature data showing a 20% increase in the detection rate of *EGFR* mutations when testing is also performed from cytologically negative samples [45]. Specifically, in our study, *EGFR* mutation testing was performed from all PE samples regardless of cytological status, and the clinical significance is reflected in the increased detection of resistant *EGFR* T790M mutation in pleural effusion samples compared to blood plasma samples (detection rate 50% in PE vs. 21.87% in blood plasma) and more patients who benefited from receiving the more effective therapy, osimertinib [46]. Repeating negative tests from blood increases the overall cost of testing, and prolongs the time-to-treatment, reducing patients' survival and quality of life. First follow-up results from our cohort on *EGFR*-mutated patients tested from PE receiving *EGFR* inhibitors reinforce the obtained data at diagnosis and progression - no progression of disease within the analyzed timeframe (unpublished clinicians' observations, awaiting a longer survival follow-up time).

Based on this data, we propose that concurrent testing of plasma and PE should be considered for *EGFR*-positive advanced non-squamous NSCLC patients whenever available to expand the number of patients that may benefit from third generation anti-*EGFR* therapy. To the best of our knowledge, these are first data of this kind from the Balkan region on the Slavic population, which are traditionally missing from larger meta-analyses. As the prognosis is known to be better in patients with *EGFR* mutations, their more accurate detection as well as the avoidance of unnecessary invasive procedures (tissue rebiopsy) contributes greatly to better patient management and a reduction the cost of optimal patient care of overall. The results might be especially significant for countries with similar, limited health resources, which have still not implemented *EGFR* mutation testing from PE.

Conclusions

Our real-world results support the current recommendations of using tissue material for initial diagnosis and reaffirm that blood plasma provides comparable and usable results as an alternative sample source when tissue is scarce or not available. At progression, superior performance of pleural effusions compared to blood plasma was shown both in the analysis of success rate and in the detection of the resistance biomarker T790M mutation. Pleural effusion is a useful analyte for *EGFR* mutation testing and might be considered in this setting whenever available, especially in countries with limited health resources.

Study approval

All analyses presented in this study are part of routine clinical diagnostics approved by the Ethics Committee of the Institute for Oncology and Radiology of Serbia and were performed in accordance with the Helsinki Declaration of 1975, as revised in 2013. All patients signed an informed consent.

CRediT authorship contribution statement

Miodrag Vukovic: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Miljana Tanic:** Conceptualization, Investigation, Methodology, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. **Ana Damjanovic:** Investigation, Methodology, Writing – review & editing. **Marijana Pavlovic:** Investigation, Methodology, Writing – review & editing. **Aleksandra Stanojevic:** Investigation, Methodology, Writing – review & editing. **Katarina Zivic:** Investigation, Methodology, Writing – review & editing. **Valentina Karadzic:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Radmila Jankovic:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Milena Cavic:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available upon reasonable request from the corresponding author. The data are not publicly available due to ethics restrictions as their containing information could compromise the privacy of patients.

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