EGFR mutation testing from liquid biopsy of non-small cell lung cancer at the Institute for Oncology and Radiology of Serbia

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Summary

Purpose: Resistance to tyrosine kinase inhibitors (TKIs) in lung cancer often occurs, so mutation testing from liquid biopsy is the method of choice as a minimally invasive approach that quickly provides information for additional therapeutic options. The purpose of this study was to assess the success rate and usefulness of EGFR testing from liquid biopsy at the Institute for Oncology and Radiology of Serbia (IORS).

Methods: EGFR mutation testing was performed by real-time qPCR in 4750 tumor samples using the Cobas® EGFR Mutation Test v2. EGFR testing from 104 liquid biopsy samples was used to track the resistance on first-line EGFR-TKIs as well as for initial testing of 124 patients without tissue biopsies.

Results: Liquid biopsy samples were tested in cases with inadequate material for DNA isolation or without tissue biopsy at diagnosis. Nine mutated samples were detected (7.3 %) with a 99.2 % testing success rate. Testing liquid biopsy samples of patients who progressed on EGFR-TKIs showed an accordance rate of 67 % with driver mutations, and 49 % of mutated patients had the T790M mutation which rendered them eligible for third-generation EGFR-TKIs. An additional 5 patients tested EGFR wild type from plasma after progression were rebiopsied and 3 of them had the T790M mutation.

Conclusion: EGFR mutation testing from liquid biopsy has been successfully implemented in Serbia and has proven invaluable for detecting molecular resistance mechanisms to EGFR-TKIs and as an alternative sample source for patients with scarce biopsy material or without any at all.

Key words: EGFR, liquid biopsy, lung cancer, personalized medicine

Introduction

With approximately 8000 newly diagnosed cases in 2018, lung cancer has become the most common malignant disease in Serbia reaching 16.5 % of all newly diagnosed cancer cases [1]. The number of deaths in 2018 was around 6800 which makes 25.3% of all cancer-related deaths and has ranked lung cancer as the most deadly cancer in Serbia [1]. These data are indicative of a strong need for better prevention measures and introduction of a lung cancer screening program, as well as its better therapeutic management and drug accessibility once it is diagnosed. The results of many recent pharmacogenomic studies overcame the old paradigm of ‘one size fits all’ in oncology, providing a large amount of molecular data that generated the concept of ‘precision medicine’. Lung adenocarcinoma has become a prominent example of precision medicine among solid malignancies.
Comprehensive genomic profiling of lung cancers revealed their genetic heterogeneity and complexity and identified numerous targetable oncogenic driver alterations [2]. These molecular profiling efforts have made it possible to exploit the potential of targeted therapies in lung cancer.

In non-small cell lung cancer, mutations in the epidermal growth factor receptor (EGFR) gene confer sensitivity to targeted therapy with tyrosine kinase inhibitors (TKIs) and their detection has been introduced as companion diagnostics at the Institute for Oncology and Radiology of Serbia (IORS) in 2011 [3,4]. The discovery of activating EGFR mutations in patients with advanced or metastatic lung adenocarcinoma who responded well to treatment with TKIs represented an essential therapeutic keystone [5,6]. Furthermore, these findings have pushed the quest for genetic alterations in other tumor types that might predict a clinical treatment benefit. Nowadays, various EGFR-TKIs are approved for use in clinical practice. First-generation EGFR-TKIs (gefitinib, erlotinib) reversibly bind to EGFR and inhibit the binding of ATP to the tyrosine kinase domain. The second-generation inhibitors, including afatinib and dacomitinib, are irreversible inhibitors, which covalently bind to EGFR [7]. Gefitinib, erlotinib and afatinib are globally approved for first-line therapy of EGFR-mutant patients. Many prospective studies have compared first-line EGFR-TKIs with standard platinum doublet chemotherapy and have confirmed superior response rates and improved progression-free survival in patients with EGFR-mutant lung cancers [8,9]. The activating mutations that confer sensitivity to first- and second-generation TKIs are also referred to as sensitizing mutations, and they reside in exons 18 through 21 of the EGFR gene [10]. In-frame deletions in exon 19 and a single point mutation in exon 21 (L858R) account for about 90% of all EGFR mutations [11]. After the approval of gefitinib in 2011, erlotinib and afatinib also became available in routine clinical practice in Serbia.

Despite the high response rates to first and second-generation EGFR-TKIs in EGFR mutant lung cancer patients, most of them inevitably acquire resistance after a progression-free period of around 10 months [12]. The secondary point mutation T790M, which substitutes methionine for threonine at amino acid position 790 of the EGFR gene, represent the most frequent mechanism of acquired resistance to first- and second-generation TKIs [13]. The T790M mutation enhances the ATP-binding affinity of EGFR-mutated cells [13]. As both first- and second-generation TKIs are competitive ATP inhibitors, their efficacy is decreased in the presence of the T790M mutation [7,14]. Third-generation EGFR-TKIs have demonstrated great potential for overcoming T790M mutation-mediated resistance [7]. Results of the AURA 2 study showed that osimertinib could be a suitable treatment for patients with T790M-positive disease who have progressed on EGFR-TKIs [15]. In 2020, osimertinib received approval for the treatment of patients with metastatic EGFR T790M mutation-positive lung adenocarcinoma. As the T790M mutation emerged as a new biomarker for patients with acquired resistance to prior EGFR-TKIs, a new challenge arose: how to perform a biopsy after resistance as it is a procedure that carries certain risks and is not always feasible [16]. A pivotal study by Oxnard et al confirmed the clinical utility of liquid biopsy, showing that patients positive for the T790M mutation in plasma had outcomes with osimertinib that were equivalent to patients who were confirmed to be positive by a tissue-based assay [17]. That way, some patients could avoid a tumor biopsy for T790M genotyping.

Mutation testing from liquid biopsy is recommended as the method of choice after EGFR-TKI resistance occurs, as it is a minimally invasive approach that quickly provides information for additional therapies [16,18,19]. In 2016 additional EGFR mutation testing was employed at the IORS from liquid biopsy samples of patients who have progressed on EGFR-TKIs, as well as for patients whose biopsies were unavailable for baseline EGFR testing.

The purpose of this study was to assess the success rate and usefulness of EGFR testing from liquid biopsy at the IORS.

Methods

Patient samples

This study included 4750 formalin-fixed and paraffin-embedded (FFPE) tumor samples or glass slides of advanced lung adenocarcinoma patients (stage IIIB/IV, ECOG performance status 0, 1 or 2) of Caucasian descent. The patients were diagnosed with primary lung adenocarcinoma according to clinical and histological criteria. FFPE lung tissue blocks were obtained by biopsy/resection and referred to the Laboratory for Molecular Genetics from various Serbian cancer centers for routine EGFR testing in the period from 2011 to 2019. Mutation testing from liquid biopsy was employed at the IORS for patients with T790M mutation status as a new biomarker for patients who have progressed on EGFR-TKIs, as well as for patients whose biopsies were unavailable for baseline EGFR testing.

The purpose of this study was to assess the success rate and usefulness of EGFR testing from liquid biopsy at the IORS.
Liquid biopsy in NSCLC

EGFR mutation testing from tissue biopsy

Genomic DNA was isolated from one to six sections of the FFPE lung tissue blocks (depending on the size of the tissue sample) using the Cobas® DNA Sample Preparation Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland). The concentration (µg/µL) and purity (A260/280 ratio) of the isolated DNA samples were determined spectrophotometrically using BioSpec-nano (Shimadzu Corp, Kyoto, Japan). EGFR mutation testing of advanced lung adenocarcinoma patients was performed by qRT-PCR using the Cobas® EGFR Mutation Test v2 on Cobas® 4800 (Roche Diagnostics).

EGFR mutation testing from liquid biopsy

In cases when tissue biopsy samples were unavailable, the presence of EGFR mutations was tested from liquid biopsy. Patients with sensitizing EGFR mutations were treated with first-line EGFR-TKIs (gefitinib, erlotinib, afatinib) until progression or unacceptable toxicity. After progression, patients were tested for the presence of resistant EGFR mutations from liquid biopsy. Testing from liquid biopsy was performed using 10µl of EDTA buffered blood that was centrifuged at +4°C for 10 min at 2000g, and the supernatant was re-centrifuged to obtain the plasma. Circulating free DNA was extracted from plasma using the Cobas® cfDNA Sample Preparation Kit and EGFR mutation testing was performed using the Cobas® EGFR Mutation Test v2 on Cobas® 4800 (Roche Diagnostics).

Statistics

Descriptive methods of statistical analysis (frequencies, percentage, mean, median, standard deviation and

Table 1. Patient and sample characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Baseline testing</th>
<th>Testing at progression on TKIs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFPE samples (n=4750)</td>
<td>Liquid biopsy (n=124)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>Male</td>
<td>2960 (62)</td>
<td>66 (53)</td>
</tr>
<tr>
<td>Female</td>
<td>1790 (38)</td>
<td>58 (47)</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>18-88</td>
<td>37-83</td>
</tr>
<tr>
<td>Median</td>
<td>63</td>
<td>61</td>
</tr>
<tr>
<td>Geographical region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vojvodina</td>
<td>1473 (31)</td>
<td>-</td>
</tr>
<tr>
<td>Central Serbia</td>
<td>3087 (65)</td>
<td>116 (93.5)</td>
</tr>
<tr>
<td>South Serbia</td>
<td>190 (4)</td>
<td>8 (6.5)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>EGFR status</th>
<th>Baseline testing</th>
<th>Testing at progression on TKIs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFPE samples (n=4750)</td>
<td>Liquid biopsy (n=124)</td>
</tr>
<tr>
<td>EGFR mut</td>
<td>514 (10.9)</td>
<td>9 (7.3)</td>
</tr>
<tr>
<td>EGFR wt</td>
<td>4231 (89)</td>
<td>114 (91.9)</td>
</tr>
<tr>
<td>NA</td>
<td>5 (0.1)</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>EGFR mutation type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex19del</td>
<td>292 (56.8)</td>
<td>6 (67)</td>
</tr>
<tr>
<td>L858R</td>
<td>155 (30)</td>
<td>2 (22)</td>
</tr>
<tr>
<td>L861Q</td>
<td>7 (1.4)</td>
<td>-</td>
</tr>
<tr>
<td>G719X</td>
<td>18 (3.5)</td>
<td>-</td>
</tr>
<tr>
<td>Ex20Ins</td>
<td>26 (5)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>S768I</td>
<td>1 (0.2)</td>
<td>-</td>
</tr>
<tr>
<td>Double mutants</td>
<td>15 (2.9)</td>
<td>-</td>
</tr>
<tr>
<td>T790M</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*concomitant with 26 samples with ex19del, 7 with the L858R mutation and 1 double mutant (G719X+S768I); **concomitant with 2 samples with ex19del and 1 with the L858R mutation
range) were used to summarize the sample data. The
Kolmogorov–Smirnov and Shapiro-Wilk tests were used
for normal distribution data testing. The associations
between the EGFR status and patient characteristics
(gender, age) and the regional distribution were ana-
lyzed using chi-square, Fisher’s exact or Wilcoxon’s test.
Two-sided p values of less than 0.05 were considered to
indicate statistical significance. The statistical analyses
were performed using GraphPad Prism (V.7.04, GraphPad
Software, CA, USA).

Results

Patient characteristics are presented in Table 1. The patient group tested from liquid biopsy at baseline consisted of 66 (53%) males, and 58 (47%) females, with patient age range of 37-83 years, and a median of 61 years. The patient group tested from liquid biopsy at progression on EGFR-TKIs consisted of 43 (41%) males, and 61 (59%) females, patient age range was 34-81 years, and the median was 60 years. The samples from both tested groups originated from two geographical regions in Serbia for which our Laboratory is the referent one for liquid biopsy testing, central Serbia (92%) and south Serbia (8%).

Baseline EGFR mutation testing

The results of EGFR mutation testing are presented in Table 2. EGFR genotyping was successfully performed in 99.9% of FFPE samples and 99.2% of liquid biopsy samples at baseline, with a turn-
around time of 5 working days. In the period from
2011-2019, 4750 EGFR mutation analyses were performed from FFPE tissue samples/glass slides, and mutations were found in 514 cases (10.9%). Testing was unsuccessful in 0.1% of the cases due to initial low sample quality/quantity, in which case re-testing from an independent FFPE sample was advised. The frequencies of mutations were 56.8% for deletions in exon 19, 30% for L858R, 1.4% for L861Q, 3.5% for G719X (G719S, G719A or G719C), 0.2% for S768I and 5% for insertions in exon 20 (Figure 1). Double mutants were detected in 2.9% of the cases, and there was no presence of the T790M mutation from tissue samples at diagnosis. Of all the detected mutations, the two most common types were ex19del and the L858R mutation which comprised a total of 87% of the mutated sample, which is in accordance with litera-
ture data [4,8]. From the mutated samples detected

![Figure 1](image1.png)

**Figure 1.** EGFR mutation presence (A) and the distribution of detected EGFR mutation types (B) in FFPE tissue and liquid biopsy samples at diagnosis (*p<0.05, **p<0.01).

![Figure 2](image2.png)

**Figure 2.** Distribution of detected EGFR mutations according to patient gender at diagnosis (A) and at progression on TKIs (B) (*p<0.05, **p<0.01).
from tissue, 38% were present in males, and 62% in females, and the obtained difference was statistically significant (p<0.001) (Figure 2). Mutation occurrence was not significantly correlated with age in any of the tested groups.

In 124 cases a tissue biopsy sample was unavailable, so EGFR mutation testing was performed from liquid biopsy (plasma). In plasma, 9 mutated samples were detected (7.3% of total) with a turnaround time of 2 working days, and a 99.2% testing success rate. The frequencies of mutations were 67% for ex19del, 22% for L858R and 11% for insertions in exon 20 (Figure 1). Double mutants were not detected in this group and there was no initial presence of the T790M mutation. The two most common types of mutations ex19del and the L858R were present in 89% of the mutated samples. From the mutated samples detected from plasma, 80% were present in females (Figure 2a).

EGFR mutation testing at progression on EGFR-TKIs

Testing liquid biopsy samples of 104 patients who progressed on first-line EGFR-TKIs showed an accordance rate of 67% with the driver mutation. The frequencies of mutations were 61.4% for deletions in exon 19, 27.1% for L858R, 2.9% for L861Q, 1.5% for G719X and 4.3% for insertions in exon 20 (Figure 3). Double mutants were detected in 2.8% of the cases. The two most common types ex19del and the L858R mutation comprised a total of 88.5% of the mutated sample. From the mutated samples detected from liquid biopsy at progression, 69% were found in female patients, and the obtained difference was statistically significant (p=0.006) (Figure 2b).

The T790M mutation was detected in 34 patients (49% of the mutated samples) which rendered them eligible for third-generation EGFR-TKIs in Serbia (Figure 3). The T790M mutation was found concomitantly with 26 samples with ex19del, 7 with the L858R mutation and 1 double mutant (G719X+S768I). The T790M was more frequently present in females (62%), but no statistical significance was reached (p>0.05). Of the 34 patients tested EGFR wild type from plasma after progression, only 5 were rebiopsied for a new tissue sample, and 3 of them were found to have the T790M mutation along with the driver mutation (2 samples with ex19del and 1 with the L858R mutation). From the additional two samples that were rebiopsied, one tested wild type and in the other the presence of the primary activating EGFR mutation only was confirmed.

Discussion

The recently reported high incidence and lung cancer-related death rate in Serbia emphasizes the need for better prevention, earlier detection and more efficient therapeutic approaches [1]. Although there has been an immense progress in treatment options in the last decade, it remains incurable in most cases, due to the fact that the majority of patients develop symptoms in advance disease stages. We and others have focused on deciphering the genetic risk and prognostic factors for lung cancer in our country, in an effort to contribute to the earlier detection and consequently better management of this disease [20-22]. However, a lung cancer screening program based on low-dose computed tomography in high-risk individuals should be introduced in Serbia as soon as possible, in order to reduce the mortality rate and increase the 5-year survival rate. Croatia is expected to be the first EU country to introduce an official nationwide screening of lung cancer in the period 2020-2024 [23].

Recognizing the importance of molecular diagnostics, the IORS established a centralized pharmacogenomics service in 2008, providing a personalized approach to the treatment of cancer patients [3,4]. After the approval of the first EGFR-TKI, gefitinib in 2011, EGFR mutation testing was implemented smoothly without major technical difficulties or delays. The same applies to liquid biopsy
testing that was introduced in 2016. Baseline EGFR genotyping was successfully performed in 99.9% of FFPE samples and 99.2% of liquid biopsy samples. EGFR mutations were detected in 10.9% of the tested patient tissue samples which is in good concordance with literature data for Caucasians. In liquid biopsy samples, 7.3% of mutated cases were found at baseline, which is lower than expected, but is still a contribution to the total pool of genotyped patients, especially important in cases when no tissue biopsy is available. There are two main reasons for the observed lower percentage of EGFR mutations from liquid biopsy. Since ctDNA comprises a small, variable fraction of total DNA circulating in the blood known as cell-free DNA (cfDNA), and mutant DNA molecules account for 0.02% to 0.1% of assayed DNA, the amount present in the tested sample is unknown [24]. Employing more sensitive methods based on emulsion PCR, such as Beads Emulsion Amplification and Magnetics (BEAMing) and droplet digital PCR (ddPCR), could overcome the issues of the low levels of ctDNA and the low allelic frequency of the variants [25].

In around half of EGFR-mutated patients tested from liquid biopsy at progression, the T790M resistant mutation was detected, which rendered them eligible for third-generation EGFR-TKIs and avoidance of chemotherapy. However, primary driver mutations were not detected in 33% of the samples. It is expected that patients who had progressed on EGFR-TKIs still have the initial driver mutation. Therefore, the T790M mutation should coexist with the primary sensitizing mutation. In cases when both the primary and the resistance mutations are not detected, it is likely that the ctDNA levels were too low for molecular analysis. Considering the possibility of false-negative results, these results should be treated as non-informative. According to the algorithm of T790M testing, tissue rebiopsy is strongly recommended in patients with a non-informative T790M plasma result [26]. Only 5 patients who tested EGFR wild type from plasma after progression were able to be rebiopsied, and 3 of them were found to have the T790M mutation along with the activating driver mutations. Additional two samples that were rebiopsied proved to have only the primary EGFR mutation or were wild type. Insufficient tissue amount and methodological limitations might explain the absence of the driver mutation in these samples. Additionally, various studies revealed spatial heterogeneity of the T790M mutation in patients with progression of disease after treatment with first- or second-generation EGFR-TKIs [27,28]. In this study, it has also been found that female patients were statistically more prone to harbouring EGFR mutations than males, and were more often carriers of the T790M resistant mutation. It has previously been reported that EGFR mutated lung cancer is more frequent in females, never-smokers, adenocarcinomas and Asian patients, thus there might be underlying genetic modifiers leading to the pathogenesis of this disease [29].

In conclusion, EGFR mutation testing from liquid biopsy at diagnosis, as well as at progression, was successfully introduced at IORS, which contributed to the most optimal management of lung cancer patients in Serbia. The technical difficulties we encountered were expected and will be taken into account for future methodological upgrading. Even though molecular profiling has traditionally relied on direct sampling of tumor tissue, blood-based diagnostics has great potential to provide clinically useful information via a minimally invasive approach. Prospective, multicentric clinical trials are warranted to further standardize the methodology and determine its precise advantages in the clinic.

Conclusions

EGFR mutation testing from liquid biopsy has been successfully implemented in Serbia and has proven invaluable for detecting molecular resistance mechanisms to EGFR-TKIs and as an alternative sample source for patients with scarce biopsy material or without any at all.

Acknowledgement

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Study approval

All analyses presented in this study are part of routine clinical diagnostics approved by the Ethics Committee of the IORS and were performed in accordance with the Helsinki Declaration of 1975, as revised in 2013.

Conflict of interests

The authors declare no conflict of interests.
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