

# Allele-specific PCR method for identification of EGFR mutations in non-small cell lung cancer: formalin-fixed paraffin-embedded tissue *versus* fresh tissue

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## Abstract

The study of epidermal growth factor receptor (EGFR) gene mutations in lung adenocarcinoma patients has a special clinical significance in the selection of patients for tyrosine-kinase inhibitor therapy. The aim of this study was to identify patients with EGFR mutations using allele-specific polymerase chain reaction (PCR), from formalin-fixed paraffin-embedded (FFPE) tissue and fresh tissue (FT). **Materials and Methods:** We performed a retrospective study using 13 cases of FFPE lung adenocarcinoma, and a prospective study using seven fresh samples of lung carcinomas (FT), collected by intraoperative dissection of the tumors. Using the DNA extracted from the FFPE tissue and FT, we attempted to identify deletions of exon 19 and point mutations of exon 21, according to the allele-specific PCR method described by Dahse *et al.* (2008). **Results:** In all seven cases of FT (three adenocarcinomas, three squamous carcinomas, one large-cell carcinoma), we identified the wild type allele and the internal control in case of exon 19, and the wild type allele for exon 21, but not the mutated alleles. Considering that no standard method for formalin fixation and paraffin embedding has been implemented at the Laboratory of Pathology, the DNA extracted from these samples became fragmented and damaged, which compromised the results of PCR testing aimed at the detection of EGFR mutations. **Conclusions:** The presented method can be implemented at our laboratory to identify these mutations from fresh tissue collected during surgical resection. Additionally, standardization of formalin fixation and paraffin embedding of surgical samples is required, in order to enable subsequent processing using molecular biology methods.

**Keywords:** allele-specific PCR, lung adenocarcinoma, fresh tissue, formalin-fixed tissue.

## Introduction

Despite recent progresses in oncology therapy, lung cancer still remains the leading causes of cancer death among men, and the third cause of death in women. One of the major breakthroughs in the study of the pathogenesis of non-small cell lung cancer (NSCLC) was the discovery of epidermal growth factor receptor (EGFR) gene mutations [1]. Published studies have demonstrated that the incidence of these mutations is higher in the Caucasian and Asian populations than in African-American populations, and it is also higher in patients with lung adenocarcinomas and females [1, 2].

Introduction of tyrosine-kinase inhibitor therapy (gefitinib, erlotinib) revolutionized the treatment of non-small cell lung cancer. Additionally, it has been shown that tyrosine-kinase inhibitors (TKIs) are beneficial especially in case of patients with EGFR gene mutations [2, 3]. The mutations that turned out to be especially reactive to TKI treatment are the in-frame deletion of codons 746–750 in exon 19, point mutation at L858R in exon 21, and point mutation at G719A/C in exon 18 [4, 5]. Approximately 90% of EGFR mutations in lung adenocarcinoma patients are the in-frame deletion of codons 746–750 in exon 19, and the point mutation at L858R in exon 21 [6]. There is a group of patients with resistance to tyrosine-kinase inhibitors. Among them, approximately 50% have substitutions in exon 20 [7].

A large number of published studies recommend

different methods for more efficient and quick identification of EGFR mutations in lung adenocarcinoma patients as compared to the standard method, direct sequencing. Many of these methods are focusing only on the most frequent mutations, and are called targeted methods. For this reason, several commercially available kits have been constructed; these provide quick and very sensitive results. Alternatively, screening methods identify more rare mutations as well, and thus the patients bearing these mutations can also benefit from TKI therapy. In case of these methods, the quality of the extracted DNA requires improvement by macrodissection or microdissection, which hinders the management of these samples [8].

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue is still a continued challenge, even after the implementation of several new methods. In case of polymerase chain reactions (PCRs) performed from such samples, the quantity of amplified DNA and the sequence length of the amplified DNA is significantly reduced, as a result of the fragmentation occurring during fixation [9].

The aim of this study was to identify patients with EGFR mutations using the allele-specific PCR method described by Dahse *et al.* [6] from formalin-fixed and paraffin-embedded tissue, and fresh tissue, not fixed with formalin, and not embedded in paraffin.

## Materials and Methods

We performed a retrospective study by processing

13 cases of lung adenocarcinomas fixed in formalin and embedded in paraffin (Cases No. 1–13, FFPE) from the material of the Laboratory of Pathology, Emergency County Hospital, Tîrgu Mureş, Romania. Important clinico-pathological parameters of these patients are presented in Table 1.

**Table 1 – Clinico-pathological parameters**

	Fresh tissue (14–20)	FFPE (1–13)
<b>Histological type</b>		
Adenocarcinoma	3	13
Squamous carcinoma	3	0
Large cell carcinoma	1	0
<b>Age [years]</b>		
Mean	60.1±9.8	60±7.3
<b>Gender</b>		
Male:Female ratio	6:1	10:3
<b>pTNM stage</b>		
pT1Nx		2
pT1N0-1		2
pT2Nx		2
pT2N0-2	3	5
pT3Nx		2
pT3N0-1	4	
<b>Smoker</b>		
Yes/No	5/2	–

FFPE: Formalin-fixed paraffin-embedded.

**Table 2 – Deparaffinization and DNA extraction conditions**

Sample No.	Tissue section	Deparaffinization		Digestion	DNA concentration [ng/μL]	A 260/280 ratio	A 260/230 ratio
		Xylene	Alcohol				
5A	3×8 μm FFPE	2×1 mL, 20 min.	2×1 mL 100%, 2×5 min.	180 μL ATL buffer + 0.1 mg proteinase K, 45°C, overnight	43.8	2.02	0.74
1-5B-12	4×4 μm FFPE	2×1 mL, 20 min.	2×1 mL 100%, 2×5 min.	180 μL ATL buffer + 0.1 mg proteinase K, 45°C, overnight	127.5	2.03	1.33
19A	25 mg FT	–	–	180 μL ATL buffer+ 0.2 mg proteinase K, 45°C, overnight	329.5	1.85	1.94
19B	25 mg FT	–	–	180 μL ATL buffer+ 0.1 mg proteinase K, 45°C, overnight	337	1.93	1.95
13A	4×4 μm FFPE	2×1 mL, 2×20 min., 21°C	2×1 mL, 100%, 70%, 50%, 2×5 min.	180 μL ATL buffer+ 0.1 mg proteinase K, 45°C, overnight	124.6	2.01	1.12
13B	4×4 μm FFPE	2×1 mL, 2×20 min., 21°C	3×1 mL, 100%, 70%, 50%, 3×5 min.	180 μL ATL buffer+ 0.06 mg proteinase K, 45°C, overnight	188.2	1.99	1.42
13C	4×4 μm FFPE	3×1 mL, 3×20 min., 21°C	3×1 mL, 100%, 70%, 50%, 3×5 min.	180 μL ATL buffer (98°C, 15 min.) + 0.1 mg proteinase K, 45°C, overnight	214	2.0	1.53
21A	25 mg FT	–	–	180 μL ATL buffer+ 0.1 mg proteinase K, 45°C, overnight	73.1	2.09	1.05
21B	4×4 μm FFPE	3×1 mL, 3×20 min., 21°C	3×1 mL, 100%, 70%, 50%, 3×5 min.	180 μL ATL buffer + 0.1 mg proteinase K, 45°C, overnight	152.7	1.99	1.43

FFPE: Formalin-fixed paraffin-embedded; FT: Fresh tissue.

Using the DNA extracted from the FFPE tissue and FT, we attempted to identify deletions of exon 19 and point mutations of exon 21, according to the allele-specific PCR method described by Dahse *et al.* (2008) [6].

PCR reactions were performed in a final volume of 25 μL for exon 19 and 20 μL for exon 21. In order to perform the reactions, we used 80–100 ng genomic DNA, 200 μmol/L dNTP (Invitrogen, Life Technologies, California, USA), and 2.5 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems, California, USA). The primers were used in the following quantities:

Deparaffinization of the sections has been performed according to the steps shown in Table 2. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen GmbH) and proteinase K (Sigma Aldrich, USA) under conditions described in Table 2. DNA concentration was measured with a spectrophotometer (Nanodrop, ND-100).

The second part of the study consisted of a prospective study that used seven samples of freshly resected lung cancers (FT, Cases No. 14–20), not fixed and not included in paraffin. These were collected by intraoperative tumor dissection performed at the No. 1 Surgery Clinic of the Emergency County Hospital, Tîrgu Mureş. Approximately 25 mg fresh tumor tissue was homogenized in 500 μL Genomic Lysis Buffer (Zymo Research) using a Potter–Elvehjem Tissue Grinder (Wheaton Science). DNA extraction was performed using the Quick-gDNA™ MiniPrep kit (Zymo Research, USA), according to the manufacturer's instructions. The DNA was diluted in 50 μL ultra purified water. We also processed a blood sample collected from a healthy patient (Case No. 146) for use as normal allele control. The molecular weight marker was 50 bp DNA ladder (Invitrogen, North America).

We took 25 mg fresh tissue from the sample of patient 19, and using different concentrations of proteinase K [0.1 mg (19B), and 0.2 mg proteinase K (19A), respectively] we extracted the DNA with the QIAamp DNA FFPE Tissue Kit, according to the manufacturer's protocol (Table 2).

0.125 μmol/L for exon 21 and 0.25 μmol/L for exon 19.

Reactions were performed according to the following steps: denaturation for 5 minutes at 94°C, followed by 30 cycles at 94°C for 0.3 minutes, annealing at 58°C (for exon 19) and 60°C (for exon 21) for 0.45 minutes, then at 72°C for seven minutes. A total volume of 10 μL of PCR reaction product was subjected to agarose gel electrophoresis (2%), followed by fixation with ethidium bromide to visualize the result under ultraviolet light.

Evaluation of the results was based on the study published by Dahse *et al.* (2008) [6]. Thus, in case of

exon 19 deletions, the homomutant allele is detected through the identification of the PB fragment, and the heteromutant allele is detected through the identification of the AQ+PB fragments (Table 3).

**Table 3 – Exon 19 (after Dahse et al. [6])**

DNA segment	Bp	Normal/ Normal	Normal/ Mutant	Mutant/ Mutant
PQ – Internal control	444	-----	-----	-----
AQ – Wild type	325	-----	-----	
PB – Mutant	134		-----	-----

For identification of exon 21 point mutations, we used two reactions: reaction T (identification of the P+A fragments, representing the wild type allele), and reaction G (identification of the P+B fragments, representing the mutant allele). In case of the wild type/mutant allele, we observe amplification of PA segments in reaction T, and that of PB segments in reaction G (Table 4).

**Table 4 – Exon 21 (after Dahse et al. [6])**

DNA segment	Bp	Reaction T	Reaction G
PA – Wild type	137	-----	
PB – Mutant	134		-----
PA + PB – Wild type/Mutant		-----	-----

The study has been performed at the Laboratory of Molecular Biology, Department of Anatomy, University of Medicine and Pharmacy of Tîrgu Mureş.

## Results

In the FT sample group, we did not identify any patient with EGFR gene mutations (deletions of exon 19 or point mutations of exon 21). This was probably due to the small number of patients included in the study, and the small proportion of adenocarcinomas in this group. Instead, we identified in all cases the internal control – PQ and the wild type allele – AQ (Figure 1) of exon 19, which proves the validity of the reaction, and the fact that it can be used on a large scale. The results correspond to those found in case of the blood sample (Case No. 146) used as normal control.

Figure 2 shows reaction T of the point mutation study

of exon 21. We observed that under ultraviolet light the wild type allele is obvious.

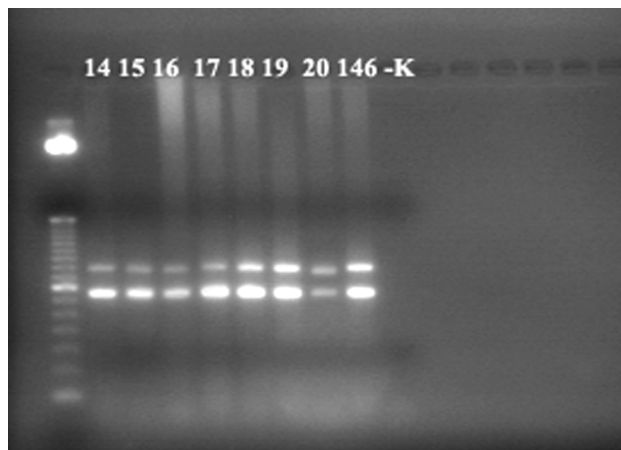
In case of reaction G, we could not demonstrate the mutant status, which shows the lack of point mutations of exon 21 (Figure 3).

Alongside DNA extraction from FT using the Zymogen kit, we also performed digestion extraction using the Qiagen kit. Quality and quantity of the extracted DNA were approximately similar (see Table 2), but after performing PCR for exon 19 mutations, only the wild type allele (AQ) showed amplification, while the internal control (PQ) did not. Diluting sample No. 19 to 80–100 ng/μL (19Bh), we observed amplification of both internal control, and wild type allele (Figure 4).

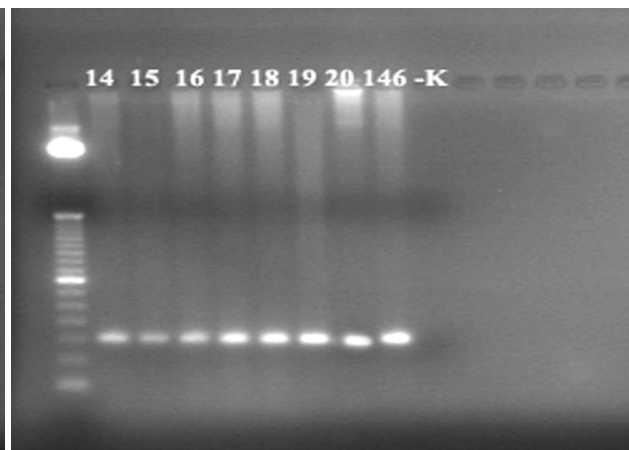
In the second part of the study, we used lung adenocarcinoma FFPE tissue. Using the conditions described in Table 2, we performed digestion extraction with the Qiagen kit. In Case No. 5A, we used 3×8 μm sections, and later in Case No. 1-5B-12, we used 4×4 μm thick sections. PCR reaction for exon 19 showed lack of amplification for both the wild type allele (AQ), and the internal control (PQ) (Figure 5).

Presuming that the FFPE tissue samples have been stored for a longer time, and under less than optimal conditions, we performed DNA extraction from a more recent case (No. 13), following the steps described in Table 2 and also varying the alcohol concentration (No. 13A); presuming that deparaffinization was incomplete we introduced an extra wash with xylene (No. 13B); finally, in order to destroy protein – DNA bonds created during fixation, we included an incubation step in lysis buffer at 98°C for 15 minutes before digestion (No. 13C). In these cases, PCR reaction for exon 19 also showed lack of amplification for both the wild type allele (AQ), and the internal control (PQ) (Figure 5).

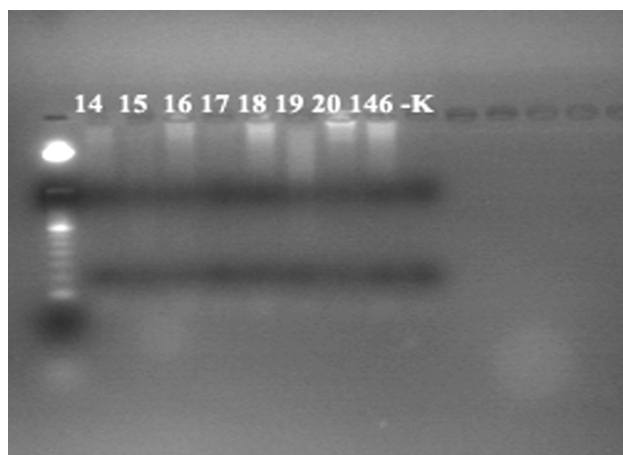
We still wanted to find out the reason of DNA deterioration and fragmentation, so we performed DNA extraction from lung cancer tissue newly fixed in formalin (25 mg) (No. 21A), followed by paraffin embedding (No. 21B), using the conditions described in Table 2. In case of these samples, PCR reaction for exon 19 showed lack of amplification for both the wild type allele (AQ), and the internal control (PQ) (Figure 5). Note that the concentration and quality of DNA was adequate in each of the cases presented in Table 2.



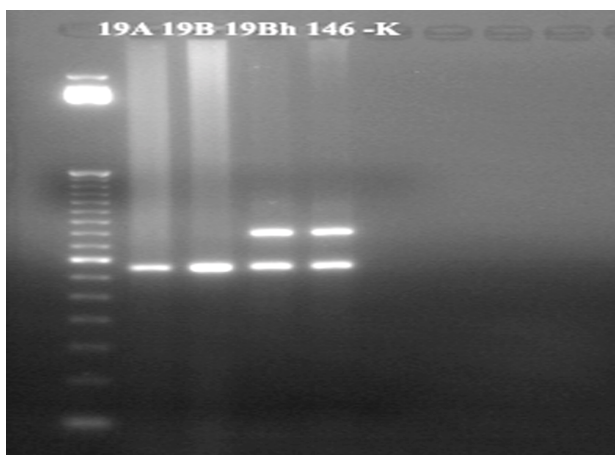
**Figure 1 – Study of exon 19. Lane 1: Molecular weight marker, FT: 14–20, 146 – normal control (blood); -K: Negative control.**



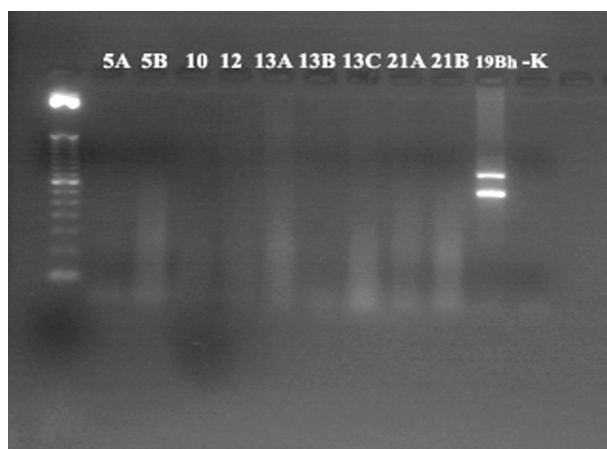
**Figure 2 – Study of exon 21, reaction T. Lane 1: Molecular weight marker, FT: 14–20, 146 – normal control (blood); -K: Negative control.**



**Figure 3 – Study of exon 21, reaction G. Lane 1: Molecular weight marker, FT: 14–20, 146 – normal control (blood).**



**Figure 4 – Exon 19, FT – Qiagen DNA extraction. Lane 1: Molecular weight marker; FT: 19A, 19B (undiluted DNA); 19Bh – diluted DNA; 146 – normal control (blood); -K: Negative control.**



**Figure 5 – Exon 19, FFPE tissue. Lane 1: Molecular weight marker; FFPE: 5A, 5B, 10, 12, 13A, 13B, 13C 21A, 21B; FT: 19Bh – normal control; -K: Negative control.**

## Discussion

In recent years, more and more studies have been focusing on the molecular biology of lung cancer [3, 10]. Of all adenocarcinoma patients, approximately 10% have EGFR mutations, and 90% of these patients display exon 19 deletions or exon 21-point mutations [11]. Approximately 16% of EGFR gene mutations in these patients involve exons 18 and 20 [3, 10].

Currently, many authors recommend implementation of routine molecular biology testing for all patients with lung adenocarcinoma, regardless of grade of differentiation, and with mixed lung cancer with areas of adenocarcinoma [1]. EGFR mutations are very rare in large cell carcinomas and adenosquamous carcinomas [11]. Identification of the mutations is recommended at the time of diagnosis [1, 2, 12]. The study of possible mutations in non-small cell lung cancer patients enables the implementation of the concept of individualized oncology therapy for these patients. Particular problems of the large-scale use of such tests are the cost of these investigations, standardization of the methods used for mutation detection, and identification of the test method that would provide adequate sensibility and specificity [2, 13].

Another important aspect to be noted is that the methods used for mutant DNA identification should be able to enable mutation identification on small sized samples, as during the diagnostic process of lung cancer, frequently only small samples are obtained by puncture (transthoracic, transbronchial) [13].

The methods used for EGFR mutation detection in lung cancer patients are divided into two categories: screening and targeted methods. Screening methods comprise direct sequencing and its alternatives. The advantages of these methods are the identification of rare mutations, and the fact that these are widespread methods. Reduced sensibility and improvement of mutant DNA content through macrodissection and microdissection, specialized equipment, technical support and long processing time are a few of the disadvantages of such methods. Targeted methods are based either on the PCR technique, and identify only deletions of exon 19 and L858R point mutation of exon 21, or on non-PCR methods, like Smart Amplification Process (SMAP). Targeted methods are faster, have a high sensibility, and identify mutations in samples with reduced tumor cell contents; their disadvantage is the exclusive identification of known mutations [2, 8].

The most widespread methods are direct sequencing and SMAP. The gold standard method, dideoxynucleotide or “Sanger” sequencing requires DNA isolation, PCR amplification and sequencing. The report is issued in a few days, but the mutant DNA has to represent more than 25% of the total quantity of extracted DNA [13]. The SMAP method requires a single step performed under isotherm conditions. The result is obtained within 30 minutes, and the quantity of the mutant DNA in the sample can be less than 0.1% [13, 14]. Other targeted methods proposed for EGFR mutation detection are length analysis, real-time PCR (RT-PCR), restriction fragment length polymorphism, high-resolution melting curve analysis, single-base extension genotyping, but any employed method has to have at least identical sensitivity to the Sanger method. It is presumed that the concordance between the results of allele specific PCR methods and the Sanger method is approximately 73% [1, 14].

Pan *et al.* (2005) proposed a simple and fast method, more sensitive than sequencing – length analysis of fluorescently labeled PCR products on capillary electro-

phoresis devices – which identifies 10% more cases with EGFR mutations compared to the gold standard method [11]. In another study published in 2008, EGFR mutations were detected by length analysis of fluorescently labeled polymerase chain reaction products and TaqMan assay from paraffin-embedded tissue, containing less than 150 tumor cells [15]. These results demonstrate that targeted methods identify mutations not detected by sequencing, even in samples with reduced mutant DNA content [8].

Two hundred to 400 tumor cells are required to identify EGFR mutations using sequencing in tumor tissue collected by biopsy. Achievement of acceptable results from formalin-fixed paraffin-embedded tissues is compromised by DNA deterioration caused by fixation and long-term storage of paraffin blocks. According to the study of Scarpino *et al.* (2015), all sections obtained from paraffin blocks contain >200 tumor cells, an average of approximately 4000 cells, and thus are appropriate for molecular testing. In case of the Sanger direct sequencing method false negative results may occur, if the rate of normal cells is significantly higher than those with EGFR mutations. Instead, for mutant specific RT-PCR less than 20% tumor cells are required to get valid results [16].

Although several studies emphasize the importance of lung adenocarcinoma patient selection for TKI treatment, and considering that first line administration of this treatment significantly improves prognosis and survival of patients with EGFR mutations, in Romania the diagnostic and treatment guideline of these patients does not recommend EGFR mutation testing [2]. Thus, the aim of this study was to implement at our laboratory the use of a simple, sensitive, low cost and fast method to identify these mutations. Consequently, we chose a highly sensitive and fast-targeted method based on a simple PCR reaction, described by Dahse *et al.* (2008) [6]. Similar methods based on allele specific PCR for EGFR mutation identification have been described by several studies [17–20], but to date we were unable to find other published studies that used the method of Dahse *et al.* [6].

According to international guidelines, good quality DNA for EGFR mutation detection can be obtained from formalin-fixed paraffin-embedded (FFPE) tissue or fresh frozen tissue (FT) collected by surgical resection, biopsy and fine-needle aspiration [2]. For PCR testing, sample fixation with 70% alcohol is recommended, but most pathology laboratories do not use alcohol as fixation agent for several reasons: tradition, cost, safety, etc. [1]. The quality of the extracted DNA is very much dependant on pre-fixation factors (type and amount of tissue, presence of autolysis) and post-fixation factors (type of fixation agent, duration, temperature, pH) [9]. The most frequently used fixation agent is 10% neutral-buffered formalin. Formalin causes formation of chemical cross-links to proteins, RNA, DNA molecules, and the DNA is fragmented if the pH is very low. Thus, in case of sequences smaller than 30 bp, amplification is not compromised, but in case of 300–1000 bp problems may occur, and reactions requiring fragments over 1000 bp cannot be performed using formalin-fixed samples. Duration of fixation is highly variable between laboratories, because it does not affect morphological detail, but extreme duration degrades DNA quality. Biopsy samples are fixed for approximately 6–12 hours, while large, surgical resection samples generally require 6–48 hours [1, 2, 9].

The classic DNA extraction protocol from paraffin-embedded tissues requires dissolving the paraffin in xylene, and consecutive washing with alcohol, because paraffin inhibits PCR amplification. Pre-digestion heat treatments can be employed (incubation at 98°C, 15 minutes, in lysis buffer) to break the cross-links between proteins and DNA, occurring because of formalin fixation [9].

While working with the 13 cases of FFPE adenocarcinomas in our study, we encountered numerous factors that influence DNA quality during pre-fixation, fixation and post-fixation. Considering that the laboratory providing the samples does not have a standardized fixation method, the time and storage conditions before fixation (transfer time of the surgical resection piece from the department to the laboratory) were different for each sample. Calcium carbonate buffered 4% formalin is used for fixation at pH 6.5–7, with a fixation time that varies very much from case to case, and frequently exceeds the recommended optimal duration (48 hours). These conditions are not recorded on the histopathology reports, considering that in Romania currently there is no recommendation for EGFR mutation testing using molecular biology methods. Thus, although we followed the DNA extraction protocol from FFPE tissue by digestion with the Qiagen kit, even trying to perform a deparaffinization (prolonging xylene incubation time, changing the alcohol concentration, introducing an incubation step at 98°C before digestion) and a more efficient digestion (changing the concentration of the enzyme), using recently collected samples fixed in formalin and embedded in paraffin (to exclude DNA damage due to storage and to establish whether fixation or paraffin embedding is responsible for DNA damage), we could not obtain a good quality DNA extract from the 13 lung adenocarcinoma cases that would enable amplification of 100–500 bp segments.

For comparison purposes with the FFPE group, we performed a prospective study using fresh lung tumor tissue obtained during surgical resection. After extracting the DNA, we performed amplification for exons 19 and 21. Upon interpretation of the results, we observed that in case of exon 19 we managed to identify the internal control and the wild type allele, and in case of exon 21 the wild type allele for all seven studied cases. Considering that currently our laboratory does not have positive and negative controls verified by sequencing, we used a blood sample collected from a healthy person as normal control for the wild type alleles (exon 19 and 21) and internal control (exon 19). In one single case, we performed comparative DNA extraction using the Zymogen and Qiagen (enzyme digestion) methods, and obtained a DNA extract that was adequate for these PCR methods. We note that for the Qiagen method, using the same amount of tissue like with the Zymogen method, regardless of the quantity of enzyme used for digestion, the concentration of the obtained DNA, dissolved in identical amount of water was much higher than with the Zymogen method. This surplus DNA, used undiluted for the PCR reaction in case of exon 19, results in amplification of only the wild type allele, and not the internal control.

The prospective study was performed on a small number of patients, due to limited access to financial resources, which resulted in inclusion of only seven patients. This is probably the cause why we were not able to demonstrate mutations of exons 19 and 21. Another reason could be

the low ratio of adenocarcinomas in this patient group (three cases of the total of seven). Instead, we successfully demonstrated the validity of the reaction and that it can be implemented at our laboratory for identification of these mutations from fresh tumor tissue.

## ✉ Conclusions

The study of EGFR gene mutations in lung adenocarcinoma patients has a special clinical significance in the selection of patients for tyrosine-kinase inhibitor therapy. Considering that in Romania currently there is no program for the selection of lung adenocarcinoma patients with EGFR mutations, in the future, we will increase the number of patients included in the study, and we will validate the first positive results by sequencing, to use these samples as positive controls for future studies. Another target would be the standardization of formalin fixation and paraffin embedding at the Laboratory of Pathology, in order to enable processing using molecular biology methods.

## Conflict of interests

The authors declare that they have no conflict of interests.

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