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Adequacy of biopsy samples for epidermal growth factor receptor (EGFR) molecular testing in lung adenocarcinoma

Adekvatnost bioptičkih uzoraka za molekularno testiranje receptora za epidermalni faktor rasta (EGFR) u adenokarcinomu pluća

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Abstract

Background/Aim. Adenocarcinoma of the lung is the most common histological type of lung cancer. The most reliable method in detecting epidermal growth factor receptor (EGFR) mutations is real-time polymerase chain reaction (PCR). It is recommended to sample three to five biopsy samples with a minimum of 200–400 preserved tumor cells. We analyzed the suitability of biopsy samples for EGFR molecular testing in lung adenocarcinoma. **Methods.** This retrospective analysis included 60 patients diagnosed with lung adenocarcinoma at the Institute for Pulmonary Diseases in Sremska Kamenica, Serbia from 2010 to 2015. Biopsy samples were obtained using transbronchial, bronchoscopic, or catheter biopsy procedures. All cases included the identification of morphometric parameters, the concentration of isolated DNA, and EGFR mutations. The proportion of tumors in biopsy samples was assessed in histological sections using computer-aided morphometry. **Results.** Biopsy samples were most commonly obtained by transbronchial biopsy (63%). In 35% of cases, there was either one or two biopsy samples. More than 10% of tumor cells were found in 68% of cases, while the majority of cases (33%) had between 200 and 500 tumor cells and only 8% of cases had between 20 and 50 tumor cells. The average concentration of DNA in all analyzed samples was 5.81 ng/μL and was significantly lower in samples provided by catheter biopsy. Only two cases with mutations were detected, and there was no statistically significant difference between the concentrations of isolated DNA in the wild type and mutated EGFR adenocarcinoma. Invalid results were found in 10% of cases. **Conclusion.** Biopsy samples are suitable for EGFR molecular testing in lung adenocarcinoma.

Key words:
lung neoplasms; adenocarcinoma; biopsy; erbb receptors; mutation.

Apstrakt

Uvod/Cilj. Adenokarcinom pluća je najčešći histološki tip karcinoma pluća. Najpouzdaniji metod za detekciju mutacija receptora epidermalnog faktora rasta (EGFR) je *real time* reakcija lančane polimerizacije (PCR). Preporuka je da se biopsijom uzme tri do pet fragmenata tkiva sa minimalno 200–400 očuvanih tumorskih ćelija. Analizirana je adekvatnost bioptičkih uzoraka tkiva za molekularno testiranje EGFR u adenokarcinomu pluća. **Metode.** Ovom retrospektivnom analizom obuhvaćeno je 60 bolesnika sa dijagnostikovanim adenokarcinomom pluća na Institutu za plućne bolesti Vojvodine u Sremskoj Kamenici, Srbija, u periodu 2010-2015. godina. Bioptički uzorci su dobijeni transbronhijalnom, bronhoskopskom ili kateter biopsijom. Kod svih uzoraka izvršene su morfometrijske analize, određena je koncentracija DNK i prisustvo EGFR mutacije. Udeo tumorskog tkiva u bioptičkom materijalu određen je primenom kompjuterskog programa za morfometrijske analize. **Rezultati.** Bioptički uzorci su najčešće dobijeni transbronhijalnom biopsijom (63%). U 35% uzoraka nalazio se jedan ili 2 bioptička uzorka. U 68% slučajeva, u uzorcima je nađeno više od 10% tumorskih ćelija, dok je najviše uzoraka imalo između 200 i 500 tumorskih ćelija, a samo 8% uzoraka između 20 i 50 ćelija. U proseku je izolovano 5.81 ng/μL DNK, a značajno niža koncentracija je utvrđena u uzorcima dobijenim kateter biopsijom. U samo dva uzorka evidentirana je mutacija EGFR, dok nije bilo razlike u koncentraciji DNK izolovane iz *wild* tip I EGFR-mutiranih karcinoma. Bilo je 10% uzoraka neadekvatnih za testiranje. **Zaključak.** Bioptički uzorci tkiva su adekvatni za molekularno testiranje EGFR u adenokarcinomu pluća.

Ključne reči:
pluća, neoplazme; adenokarcinom; biopsija; erbb receptori; mutacija.

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Introduction

Lung carcinoma (LC) is the leading cause of morbidity and mortality of malignant diseases in the world [1, 2]. Approximately 80% of patients with LC have unresectable tumors at the time of diagnosis. A new approach in the treatment of patients with lung adenocarcinoma (ADC) and epidermal growth factor receptor (EGFR) mutations are associated with sensitivity to the EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib [3–7].

Surgical samples represent the gold standard for EGFR molecular testing, but formalin-fixed paraffin-embedded biopsy samples, cytological samples, and blood plasma may also be used for this testing. The material obtained by any of these methods is considered adequate for testing [8]. The diagnostic accuracy and adequacy of the biopsy samples depend on their diagnostic modality, and the diameter of the needles used [9–14]. To date, there has been no consensus on the number of tumor cells (TC) necessary for EGFR molecular testing. According to Travis et al. [15], the recommendation for EGFR testing is a minimum of 200 to 400 TC.

Polymerase chain reaction (PCR) is the most widely used and most reliable method for determining EGFR mutations since it requires a minimum amount of the starting material and allows amplification of the desired DNA segment up to a billion times [16]. The PCR method is carried out in three basic steps: denaturation of double-stranded DNA matrix, hybridization of specific oligonucleotides (primers) and DNA matrix, and the extension (elongation) of primers that comprise a single PCR cycle repeated 20 to 45 times, where the desired DNA fragment is multiplied by a million to a billion times. After 40 repeated cycles, the efficiency of the reaction is lost, and the "plateau phenomenon" occurs [17].

The aim of this research was to determine the adequacy of the biopsy samples obtained by different biopsy procedures for EGFR molecular testing in lung ADC.

Methods

Pathohistological analysis and formation of groups

Sixty cases of primary ADC of the lungs diagnosed by biopsy samples were obtained from the records of the Institute for Pulmonary Diseases of Vojvodina from January 2010 to December 2015. The tissue was obtained during transbronchial biopsy (TBB), bronchoscopic biopsy (BB), or catheter biopsy (CB). The original diagnostic hematoxylin and eosin-stained sections were reviewed by two pathologists independently.

The number of biopsy samples (tissue fragments obtained during the biopsy procedure) and the number of TC in the whole biopsy sample were recorded using the Olympus BX43 (Olympus, Tokyo, Japan) light microscope with a magnification of 100X and 400X. All samples were classified into groups according to the number of TC: group I (< 20), group II (21–50), group III (51–100), group IV (101–200), group V (201–500), group VI (501–1000), and group VII (> 1000).

Computer-aided digital morphometry was used to determine the volume densities of tumor tissue (Tvd), non-tumor tissue volume density (NTvd), blood (Bvd), and necrosis (Nvd).

This was performed using the "Olympus DP73" digital camera (Olympus, Tokyo, Japan) that was attached to the Olympus BX43 light microscope. Digital images of the biopsy sample were captured at high power using a 40X lens and then analyzed using the "Image J" software with an installed plugin for the analysis of the number of hits (COST and Analyze). The total biopsy sample, composed of non-tumor tissue (NT) and tumor tissue (T), was digitally covered by a predefined number of hits using the program mentioned above (Figure 1). Given that the volume density of the entire biopsy sample accounts for 100%, based on the obtained data on the number of hits on the entire biopsy (Bih), tumor tissue (Th), non-tumor tissue (NTh), blood (Bh), and necrosis (Nh), Tvd, NTvd, Bvd, and Nvd (in percentages) were calculated using the proportions method (100% : Bih = Tvd : Th; 100% : Bih = NTvd : NTh; 100% : Bih = Bvd : Bh; 100% : Bih = Nvd : Nh).

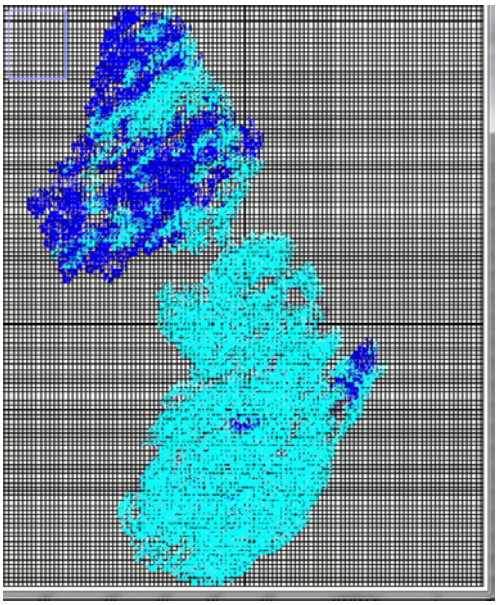

Fig. 1 – Morphometric analysis of biopsy sample (with lung adenocarcinoma tissue and non-tumor tissue) using Image J program [blue – tumor cells (TC); cyan – non-TC; ×40].

Real-time PCR (rtPCR) analysis

Molecular analysis of the EGFR mutation (exon 18-21) was done prospectively for all cases with the cobas EGFR Mutation Test (Roche, Basel, Switzerland) rtPCR. The cobas Sample Preparation Kit *(*Roche, Basel, Switzerland) was used for sample preparation and DNA extraction. Automatic amplification and detection were done on the cobas z 480 Analyzer (Roche, Basel, Switzerland).

Statistical methods

Assessment of correlations and comparisons between the mean values of numerically expressed data groups was performed using the *t*-test and analysis of varience (ANOVA) methods.

Statistical analysis was performed using SPSS 12.0 software.

Results

General characteristics of the patients are shown in Table 1. The median number of biopsy samples verified microscopically was two, with a minimum of 1 and a maximum of 7 samples per slide. More than half of the samples contained one or two biopsy samples. The cases with three, four, and seven samples were rare (20%, 2%, and 2%, respectively), and 4 among 60 analyzed cases could not be classified.

Table 1

Characteristics of patients

Characteristics	Values
Total number, n (%)	60 (100)
Gender, n (%)	
men	35 (58)
women	25 (42)
Age (years), mean ± SD (range)	61.8 ± 8.08 (43–79)
Smoking status, n (%)	
non-smoker	6 (10)
former smoker	10 (17)
smoker	44 (73)

Considering the number of TC, the majority of cases were classified into Group V (33%) and the minority in Group I (0%) and II (8%) (Table 2).

Table 2

Data on type of biopsy performed, and number of tumor cells in one sample

Type of biopsy	Number of cases, n (%)	Tvd, %
TBB	38 (63)	21.63
BB	10 (17)	31.22
CB	12 (20)	21.08
Number of TC		
I (< 20)	0 (0)	0
II (21–50)	5 (8)	6.64
III (51–100)	10 (17)	10.95
IV (101–200)	12 (20)	10.87
V (201–500)	20 (33)	31.63
VI (501–1000)	13 (22)	37.70
VII (> 1000)	0 (0)	0

TBB – transbronchial biopsy; BB – bronchobiopsy; CB – catheter biopsy; TC – tumor cells; Tvd – volume densities of tumor tissue.

Analyzing the biopsy samples, the median value of the Tvd was 24.88%, and there was no statistically significant difference in comparing the Tvd according to the type of the performed biopsy procedure (TBB, BB, CB) ($p = 0.360$). What is perhaps even more significant was a statistically significant difference in the number of TC among the samples obtained during different biopsy procedures when we compared the samples with small Tvd (less than 10%) ($p < 0.001$). In those samples with low Tvd, the biopsy samples obtained during CB had a significantly lower number of TC. Necrosis was recorded in two cases, with Nvd of 20% and 40%, but valid EGFR molecular testing results were obtained. Blood was found in 20 out of 60 cases, and Bvd ranged from 2.25% to 90.72%. In two of the cases containing blood (with Bvd values 11.7 and 71.7), the results of molecular testing were invalid, even after retesting. Out of all analyzed cases, six of them (10%) were invalid even after retesting. The average concentration of DNA was 5.81 ng/μL (range 0.38–19.2 ng/μL), and it was significantly lower in samples provided by CB.

EGFR mutations were detected in two cases, both of them women, one of which was a non-smoker. The tissue in EGFR mutated ADC cases was obtained by TBB, and according to the number of TC, the samples were put into group III. Tvd of one of the mutated cases was 11.59%, and the other had Tvd < 10%. One case had blood (Bvd = 32%), while necrosis was absent in both cases. There was no statistically significant difference in comparing the concentration of DNA in wild type (*wt*) and EGFR mutated ADC ($p = 0.641$). In cases with mutations detected, an average concentration of DNA was 4.53 ng/μL.

Discussion

ADC has been the most common histological type of LC in the last few decades, more frequently in men [18–25]. This fact was also confirmed by the results of our research, in which the ratio of affected men and women was 1.4 : 1. The average age of patients was 61.8 ± 8.08 years. Smoking is one of the most important risk factors for the development of LC [26–29]. The high percentage of active smokers in our research is most likely the consequence of poor socio-economic status, advocating bad lifestyles and bad effects of smoking ban campaigns.

Bronchoscopy is safe and well-tolerated by the patients and has become the mainstay investigation in the evaluation of patients with LC and may be used for molecular biologic analyses to help select therapy and provide prognostic information. The sensitivity and specificity of the biopsy samples depend on the location and distribution of the tumor and the number of samples obtained during the biopsy procedure [30–33]. The amount of tumor tissue obtained by biopsy is small since the obtained tissue contains both tumor and non-tumor cells. Moreover, the amount of tumor tissue depends on the histological type of the tumor and endoscopic findings. The presence of necrosis or the presence of crush artefact (even in a visible endobronchial disease) may cause the failure in achieving the histological diagnosis. In these circumstances, a combination of different cytological and histological procedures provides the optimum diagnostic yield. The number of biopsy samples in published papers varies, although it is recommended to take 3 to 5 samples [25, 31, 32]. Bronchoscopy has been implemented on our Institute since 1960. In this research, we recorded an average of two biopsy

samples (range 1–7) during one procedure, which is a significantly lower number in comparison to the results published.

The majority of biopsy samples contained more than 100 TC [25]. Similar to the above results, more than half of the biopsy samples included in our research had more than 100 TC. There are various methods for the morphometric analysis of biopsy samples. In the analysis of 120 cases, Scarpino et al. [33] used digitized slides where the following items were determined manually: biopsy area, tumor area, TC number, and the total number of cells in the biopsy, followed by determining the percentage of tumor tissue and TC.

In this research, Tvd was determined using the Image J computer program with installed plugins for the analysis of the number of hits (COST and Analyze). Tvd depends on the type of the tested sample and how the samples were obtained. The average Tvd value in our research was 24.88%. After comparing Tvd among the samples obtained with different types of biopsy procedures using the ANOVA method, the obtained difference was not statistically significant ($p = 0.36$). A significant number of biopsy samples (32%) in our research had Tvd less than 10%, which is contrary to the results of Zhu et al. [25] (4.7%).

The concentration of isolated DNA does not differ among the patients with *wt* EGFR and the mutated EGFR ADC [33, 34]. This view was confirmed by the results of our research ($p = 0.641$). In a study by Scarpino et al. [33], there was no statistically significant difference observed among the samples obtained by transthoracic puncture and biopsy (8.0 ng/μL vs. 9.2 ng/μL). Contrary to these results, we have found that the concentration of isolated DNA depends on the type of biopsy procedure and that there is a statistically significant difference between the samples obtained by BB and CB ($p = 0.055$).

The number of biopsies with an insufficient amount of DNA for molecular EGFR testing depends on the quality of the material analyzed. Khode et al. [35] identified an insufficient amount of extracted DNA in 6 (11%) out of 56 cases and recommended resampling. Although blood and necrosis may be the limiting factors for molecular EGFR testing, they were not the exclusion factors in our research, and the percentage of invalid results of 10% was in line with the results of the previous study. Necrosis was recorded in 2 cases only. Blood was present in 20 cases, but only 2 of them had invalid results, even after retesting. The presence of these factors was not considered an absolute limiting factor for exclusion from the EGFR testing.

In detecting mutations, biopsy and cytological samples are used equally with surgical samples, thus eliminating the need for invasive diagnostic procedures [34]. EGFR mutations can also be demonstrated in biopsy samples with a small number of TC [36]. This view was confirmed by the results obtained by Krawczyk et al. [34]. They detected a similar percentage of EGFR mutations in the biopsy samples with TC < 20% and TC ≥ 20% (8.1% vs. 9.2%) [34]. Contrary to these results, Scarpino et al. [33] recorded a smaller percentage of EGFR mutations in biopsy samples with TC < 20% as compared to samples with TC ≥ 20% (19% vs. 25%) ($p > 0.05$) [33]. If the comparison limit is 50% of TC, the difference is statistically significant [24]. Both cases with EGFR mutated ADC in our research had less than 20% of TC, which contributed to the understanding that EGFR mutation can also be determined in biopsy samples with a small number of TC.

In the first major study on the EGFR mutation status of patients from Serbia, EGFR mutations were detected in 42/360 (11.7%) patients with lung ADC [37]. Contrary to these results, we detected EGFR mutations in a significantly smaller percentage (2/60; 3.3%). Deletions in exon 19 are most often detected by applying the Cobas® EGFR Mutation Test [35, 37]. We did not detect this type of mutation, and the results of our research are most likely the consequence of a smaller number of patients involved in the research, as well as a smaller percentage of EGFR mutated lung ADC.

Conclusion

Based on our data presented here, we think that BB is suitable for EGFR molecular testing in lung ADC.

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