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**Association between EGFR mutation status, clinicopathological characteristics and TTF-1 expression in lung adenocarcinoma – a single center study**

Удруженост мутационог статуса *EGFR* са клиничкопатолошким карактеристикама и *TTF-1* експресијом у аденокарциному плућа – студија једног центра

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## Association between EGFR mutation status, clinicopathological characteristics and TTF-1 expression in lung adenocarcinoma – a single center study

Удруженост мутационог статуса *EGFR* са клиничкопатолошким карактеристикама и *TTF-1* експресијом у аденокарциному плућа – студија једног центра

### SUMMARY

**Introduction/Objective** The presence of EGFR mutations is the best predictor of response for therapy with tyrosine kinase inhibitors. In this study we investigate association between EGFR mutations and clinicopathological characteristics and thyroid transcription factor (TTF-1) expression in lung adenocarcinomas (ADs).

**Methods** We analyzed 142 surgical samples from patients with histologically confirmed lung ADs from January, 2010. to December, 2015. All tumor tissues were reclassified according to WHO criteria and EGFR mutations detected by Real time PCR. TTF-1 expression was detected by immunohistochemistry in 83 out of 142 cases. The association between EGFR and TTF-1 expression was analyzed using  $\chi^2$  test or Fisher's exact test with SPSS software version 20.0.

**Results** This study included 78 male and 64 women with a median age 61.6 (range, 42-82) years. Acinar (ACN) and solid (SOL) were the most common histological types (47.9% and 38.7% respectively). TTF-1 expression was present in 69 of 83 (83%) ADs. The EGFR mutation was found in 7%, more frequently in women, and patients with smoking history, and acinar type of ADs, whereas it had no association with age and pathological stage and TTF-1 expression.

**Conclusion** In conclusion, the results of this study demonstrate that the presence of EGFR mutations is associated with some clinical characteristics and histologic type of ADs, but not with TTF-1 expression.

**Keywords:** adenocarcinoma; EGFR mutation; clinicopathological characteristics; TTF-1 expression

### САЖЕТАК

**Увод/Циљ** Присуство *EGFR* мутација је најбољи предиктор одговора на терапију инхибиторима тирозин киназе. У овој студији смо истраживали удруженост *EGFR* мутација са клиничкопатолошким карактеристикама и експресијом *Thyroid Transcription Factor-1 (TTF-1)* у аденокарциномима плућа (АД).

**Метод** Анализирана су 142 хируршка узорка пацијената са хистолошки потврђеним АД плућа у период од јануара 2010. до децембра 2015. године. Сви туморски узорци су рекласификовани према критеријумима СЗО и код свих су *EGFR* мутације детектоване методом *PCR* у реалном времену. *TTF-1* експресија је одређена имунохистохемијски у 83 од 142 случаја. Асоцијација *EGFR* мутација и *TTF-1* експресије је анализирана употребом  $\chi^2$  теста или Фишеровог теста.

**Резултати** У студију је било укључено 78 мушкараца и 64 жене просечне старости 61.6 (од 42 до 82) година. Ацинарни и солидни типови су били најчешћи хистолошки типови (47.9% и 38.7%). Експресија *TTF-1* била је присутна у 69 од 83 (83%) АД. Мутације *EGFR* детектоване су у 7% случајева, чешће код жена, бивших и активних пушача, са ацинарним хистолошким типом и нису биле повезане са годинама, патолошким стадијумом болести и експресијом *TTF-1*.

**Закључак** Резултати ове студије показују да је присуство мутација *EGFR* удружено са неким клиничким карактеристикама и хистолошким типом АД, али не и са експресијом *TTF-1*.

**Кључне речи:** аденокарцином; мутације *EGFR*; клиничкопатолошке карактеристике; експресија *TTF-1*

### INTRODUCTION

Epidermal growth factor receptor (EGFR) consists of 486 amino acids and 170 kDa in size. It is part of the ErbB family of structurally related receptor tyrosine kinases: EGFR (HER1, ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3) and HER4 (ErbB4) which are involved

in signal transduction pathways and play a key role in the regulation of cellular proliferation and apoptosis. Molecular analysis of the mutation status for EGFR is critical for treatment of tyrosine kinase inhibitors (TKIs), which show improved progression-free survival (PFS) and overall survival in patients with ADs which is second most frequent histological type of lung cancer found in surgically treated lung cancer patients in Serbia [1, 2, 3]. According to international guidelines, conventional identification of EGFR genotype requires tissue/cytologic samples (Ti/Cy), but in last five years EGFR testing can be performed by analysis of circulating-free tumor DNA (cfDNA) in peripheral blood samples. EGFR mutations are more frequent in tumors with ADs histology, in never-smokers or light smokers, in female, and in patients with East Asian ethnicities. The most frequent EGFR mutations are in-frame deletions of exon 19 and the exon 21 L858R mutation [4, 5]. Previous investigations have demonstrated that EGFR gene mutation is mainly detected in patients with lepidic (LP), papillary (PAP), micropapillary (MPP) and acinar (ACN) types, whereas the mutation rate is extremely low in patients with solid (SOL) histological type [6, 7, 8, 9].

Thyroid transcription factor 1 (TTF-1), is a homeodomain nuclear protein that belongs to the NK2 family of transcription factor. TTF1 is recommended as one of a panel of lineage-specific immunohistochemical markers for ADs differentiation and may modulate lung cancer biology. Clinicopathologic features such as age, gender, and smoking status, histological type and pathological stage were similar between TTF-1 positive and TTF-1 negative tumors. TTF-1 positive tumors have more commonly EGFR mutations, as well as better response to EGFR TKIs comparing to TTF-1 negative tumors, but TTF-1 negativity should not be the exclusion criteria for EGFR testing [10, 11, 12].

In the present study, we investigated the association between EGFR mutation status, clinicopathological characteristics and TTF-1 expression in lung adenocarcinoma.

## METHODS

The surgical samples of 142 patients with lung ADs admitted to the Institute for Lung Diseases of Vojvodina (Sremska Kamenica, Serbia) between January, 2010. and December, 2015. were retrospectively analyzed.

Clinicopathological parameters including age, gender, smoking history, pathological stage and histological type were recorded. The histological classification was done based on 2015 WHO classification system and all samples were divided in five groups (ACN, PAP, MPP, LP or SOL).

142 surgical samples were fixed in 10% formalin, embedded in paraffin, cut on four-micron-thick sections and stained with routine H&E staining. Eighty-three of them were deparaffinized and incubated in a citrate buffer (10 mM sodium-citrate monohydrate, pH 6.5) at 120°C for 20 min in an autoclave. The sections were reacted for 1 hour with antibody of TTF-1 (monoclonal antibody, Denmark DAKO products) and then incubated with a commercially available detection kit (DAKO EnVision Plus-HRP, Dako, Glostrup, Denmark) following the manufacturer's instructions. Positive and negative controls were used as appropriate. TTF-1 expression was rendered semiquantitatively on a score from 0 to 2. Tumors were scored according to TTF-1 expression into following scores: 0 (lack of expression), score 1 (< 50%) and score 2 (more  $\geq$ 50%) based on the percentage of positively stained tumor cells of any intensity.

The EGFR mutations (in exons 18, 19, 20, and 21) was done with the Cobas EGFR Mutation Test (Roche, Basel, Switzerland) "Real time PCR". The Cobas Sample Preparation Kit (Roche, Basel, Switzerland) was used for the sample preparation and DNA extraction. Automatic amplification and detection were done on the Cobas z 480 Analyzer (Roche, Basel, Switzerland).

### **Statistical analysis**

Pearson's  $\chi^2$  test and Fisher's exact test were used to compare frequencies of clinicopathological variables. P-values of < 0.05 were considered significant. All analyses were performed using SPSS version 20 (SPSS, Chicago, IL, USA).

The study was done in accord with standards of the institutional committee on ethics.

## RESULTS

One hundred and forty-two patients diagnosed with infiltrative ADs (Figure 1) which had been surgically resected were included in this study. The median age of all patients was 61.6 years (ranging from 42 to 82). The majority of patients were males (78; 54.9%), and 13 of 142 (9.2%) had no-smoking history (Table 1).

Most common histological types were ACN (68; 47.9%) and SOL (55; 38.7%) with most frequent pathological stage IIA according to the 7<sup>th</sup> TNM classification system (Table 2).

Sixty-three (75.9%) of the 83 cases of lung ADs showed TTF-1 expression levels corresponding to score 2 (Figure 2), 3 (7.2%) to score 1 and 14 (16.9%) cases lack of TTF-1 expression (Table 3).

EGFR mutations in exons 18, exon 19, exon 20 or exon 21 were examined in 142 cases of lung ADs. The overall frequency of EGFR mutation was 7% (10 of 142). Five cases had an in-frame deletion in the exon 19, three cases had exon 21 substitutions (L858R, L861Q) and two cases exon 20 insertions. Multiple mutations ( $\geq 2$ ) and other EGFR mutations were not observed (Figure 3).

Three male and seven female patients harbored EGFR mutation ( $P = 0.114$ ). Three patients with EGFR gene mutation were non-smokers. ( $p = 0.05$ ) (Table 1). The EGFR mutations were found in eight ADs with ACN histological type and two with SOL histological type ( $p = 0.32$ ). Other histological types were without EGFR mutations. Pathological stage with EGFR mutations were: IIB, IIA and IB (4, 3 and 1;  $p = 0.46$ ) (Table 2). Association between TTF-1 protein expression and EGFR mutation status is shown in Table 3. Three cases with TTF-1 expression (score 2) had EGFR mutations. Also, EGFR mutations were present in two cases lack of TTF-1 expression (score 0) (Table 3).

## DISCUSSION

In recent years, significant progression has been obtained in the molecular biological research of lung ADs with EGFR mutation. EGFR mutation is a protein on cell surface with intracellular tyrosine kinase (TK) activity due to targetable activating mutations. These tumors are susceptible to TKIs such as gefitinib, erlotinib, or afatinib. [9]

Ti/Cy samples are mostly obtained by sampling of primary tumor or metastatic lymph node. The median EGFR test turnaround time for Ti/Cy samples was 11 days for Europe and 8 days for Japan. When tumor samples are unavailable, cfDNA is a feasible sample for EGFR mutation analysis because overall concordance of EGFR mutation status between matched Ti/Cy and plasma samples was 89%. It is important to conduct mutation testing in specialized laboratories, using sensitive mutation testing methods to ensure accuracy of results. In Europe 43 laboratories perform the Ti/Cy testing, while in five laboratories both analysis of Ti/Cy and cfDNA in peripheral blood samples are performed [11, 13, 14]. At our institution, both methods of EGFR mutation testing have been implemented in clinical practice three years ago.

Since CT is routinely used in lung cancer diagnosis, as CT imaging is more readily available than biopsies, many researchers proposed analysis CT imaging for predicting EGFR mutations [15]. The presence of emphysema or airway abnormality predicts a wild type status of EGFR while the presence of any ground glass component indicates EGFR mutations [16]. Recently, the deep learning model (DLM) provides a non-invasive and easy-to-use method for prediction of EGFR mutation status. Wang et al. retrospectively collected data from 844 lung ADs patients with pre-operative CT images, EGFR mutation and clinical information from two hospitals. The DL score demonstrated significant differences in EGFR-mutant and EGFR-wild type tumors ( $p < 0.001$ ) [17].

ADs are the most common histological subtypes of lung carcinoma, and it has been shown that is associated with activating mutations in the EGFR gene in 15% of European and 47% of Japanese patients [18]. The most common EGFR mutations include exon 19 deletion. Other recurrent mutations, in exon 18-point mutations in position G719, in exon 21 L861Q mutation, and in-frame exon 19 insertions are rare (3%; 2% and  $< 1\%$ ) [19, 20, 21, 22]. In our

study EGFR mutations were detected in 10 of 142 (7%) cases ADs, five of ten were deletion in exon 19, similar with results in other studies.

The association between EGFR mutations with patient's age, gender and smoking status have been demonstrated in numerous studies with a variety of cases [23, 24, 25, 26]. In this investigation, EGFR gene mutation was mainly observed in patients aged > 60 years, which is consistent with previous findings.

Mutations were found more frequently in women (69.7%), in patients who had never smoked (66.6%), and in those with adenocarcinomas (80.9%) ( $p < 0.001$ ) [22, 26]. Contrary to these results, in our study EGFR mutations were more frequently detected in smokers (7 of 10 cases). These differences are probably the result of a different lifestyle.

Many authors have studied the association between EGFR mutations and histologic type of Ads and TTF-1 expression, as well as pathologic stage of the disease [27, 28, 29, 30]. Villa et al. reported that the most common histologic type seen in the EGFR-mutant-positive ADs was LP (44%) [31]. Contrary to these results, Zhang et al. reported that ACN type most frequently correlated with EGFR mutation, which is consistent with our results [22]. In our study, ACN (7/10) and SOL (3/10) type were independent predictors of EGFR mutation. EGFR-mutated ADs may develop through a distinct carcinogenetic pathway, in which the MPP element may play an important role in promoting progression and has prognostic value [32]. In our results MPP histologic type was detected in 4 of 142 (2.8%) cases with wild type EGFR ADs. Pi et al. showed that EGFR mutation was significantly higher in stage IA than in stage IIB ( $p = 0.002$ ) [27]. In our study, there was no difference in EGFR mutations between stage IA and stage IIB, probably because it was single center study with small number of cases.

TTF-1 is expressed in the distal bronchial epithelium, including type II alveolar epithelial cells and terminal respiratory epithelial cells, as well as in lung carcinoma: frequently in small cell carcinoma and in ADs [10]. Sixty-six of the 83 cases (79.5%) of lung ADs included in this study showed score 2 (75.9%) of TTF-1 expression, while 3 (7.2%) showed score 1. These results suggest that lung ADs expressed TTF-1. In recent years, many studies have mentioned the association between TTF-1 expression and EGFR mutations [10, 12, 32, 33]. The TTF1 positivity staining were strongly correlated with the presence of EGFR

mutations ( $p < 0.001$ ) and TTF-1 negativity to be a good predictor of EGFR wt mutations [32]. The results of the Svaton et al. study suggested that patients with EGFR wt lung ADs and lack of TTF-1 expression to have significantly lower PFS and OS, and TTF1 expression may be useful predictors of TKIs efficacy in patients with EGFR wt lung ADs [29]. The patients, TTF-1 positive or negative ADs, could benefit from the first-line chemotherapy [30]. Therefore, lack of TTF-1 expression should not exclude patients from EGFR testing.

To our knowledge, this is the first study which investigate association between EGFR mutation and TTF-1 expression in Serbia. Among tumors in which immunohistochemical analysis to TTF-1 antibody was performed ( $n = 83$ ), EGFR mutations were detected in five cases: in three tumors with score 2 of TTF-1 expression and in two tumors lack of TTF-1 expression (Table 3).

## CONCLUSION

The results of this study demonstrate that the presence of EGFR mutations is associated with some clinical characteristics and histologic type of AD, but not with TTF-1 expression.

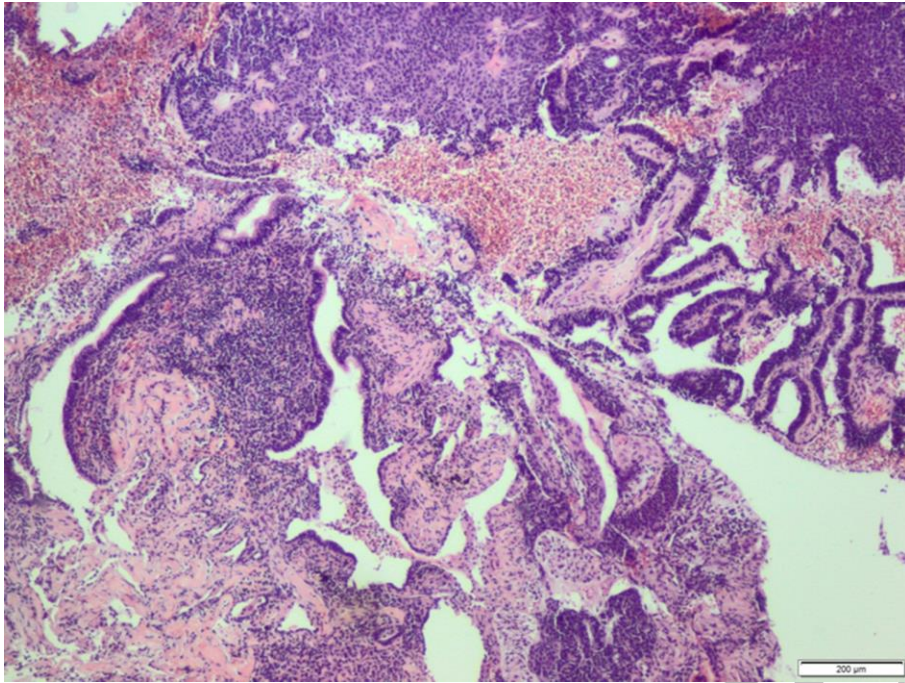
**Conflict of interest:** None declared.



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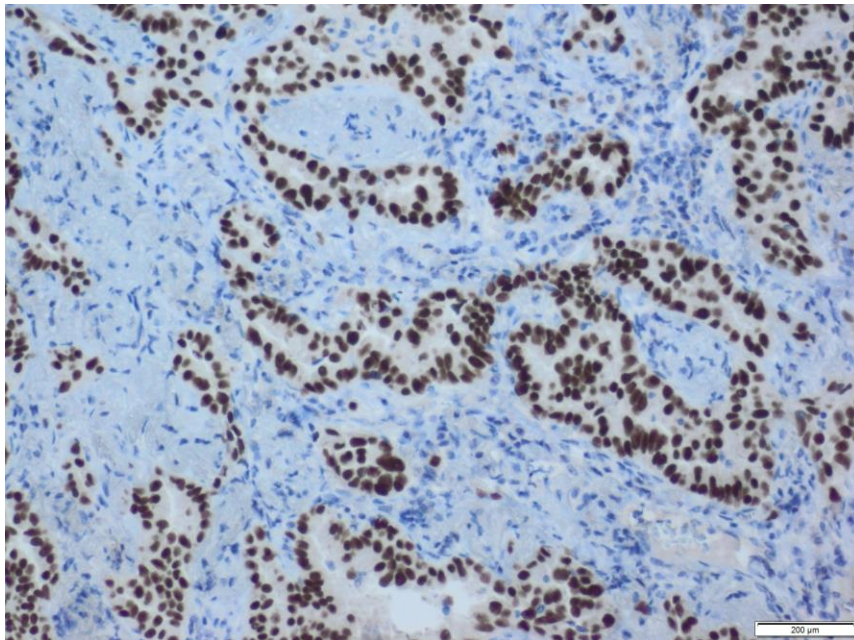
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**Figure 1.** Acinic type adenocarcinoma (H&E, 10×)

Paper accepted



**Figure 2.** TTF-1-positive expression in adenocarcinoma cells (IHC, 10×)

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**Table 1.** Patients characteristics and epidermal growth factor receptor (EGFR) mutation status

Clinical characteristics	Frequency	EGFR status n (%)		p
		positive	negative	
Total	142	10 (7)	132 (93)	
Age, years				
Median	61.6	65.5	61.3	
Range	42–82	46–82	42–82	
Sex				
Male	78 (54.9)	3 (2.1)	75 (52.8)	0.114
Female	64 (45.1)	7 (4.9)	57 (40.1)	
Smoking history				
Never	13 (9.2)	3 (2.1)	10 (7)	0.05
Former/current	129 (90.8)	7 (4.9)	122 (85.9)	

**Table 2.** Histopathological characteristics of adenocarcinomas and epidermal growth factor receptor mutation status

Histopathological characteristics	Frequency	EGFR status, n (%)		p
		positive	negative	
Histological type				
LP	6 (4.2)	0 (0.0)	6 (4.2)	0.32
ACN	68 (47.9)	8 (5.6)	60 (42.3)	
PAP	9 (6.3)	0 (0.0)	9 (6.3)	
SOL	55 (38.7)	2 (1.4)	53 (37.3)	
MPP	4 (2.8)	0.(0.0)	4 (2.8)	
Stage				
IA	32 (22.5)	3 (2.1)	29 (20.4)	0.46
IB	24 (16.9)	1 (0.7)	23 (16.2)	
IIA	35 (24.6)	2 (1.4)	33 (23.2)	
IIB	28 (19.7)	4 (2.8)	24 (16.9)	
IIIA	21 (14.8)	0 (0.0)	21 (14.8)	
IV	2 (1.4)	0 (0-0)	2 (1.4)	
Total	142 (100)	10 (7)	132 (93)	

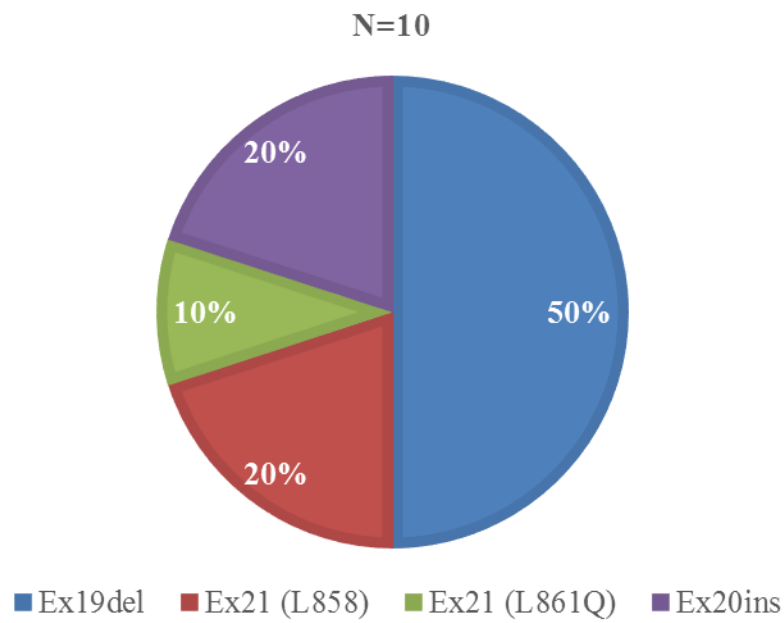
EGFR – epidermal growth factor receptor; LP – lepidic; PAP – papillary; MPP – micropapillary; SOL – solid; ACN – acinar

**Table 3.** Association between TTF-1 expression and epidermal growth factor receptor mutations

TTF-1 expression	Frequency	EGFR status, n (%)	
		positive	negative
Score 0	14 (16.9)	2 (2.4)	12 (14.5)
Score 1	3 (7.2)	0 (0)	6 (7.2)
Score 2	63 (75.9)	3 (3.6)	60 (72.3)
Total	83 (100)	5 (6)	78 (94)

EGFR – epidermal growth factor receptor

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**Figure 3.** The proportion of epidermal growth factor receptor mutations