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Evaluation of epidermal growth factor receptor mutations and thyroid transcription factor-1 status in Turkish non-small cell lung carcinoma patients: A study of 600 cases from a single center

Küçük hücreli dışı akciğer karsinomlu Türk hastalarda epidermal büyüme faktör reseptör mutasyonları ve tiroid transkripsiyon faktör-1 durumunun değerlendirilmesi: Tek merkezli 600 hastalık çalışma

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ABSTRACT

Background: This study aims to investigate the frequency, distribution, and morphological/immunohistochemical features of epidermal growth factor receptor mutations and to examine the possible relationship between the material type and technical success of mutation analysis in Turkish population with non-small cell lung cancer.

Methods: Between September 2012 and December 2015, a total of 499 consecutive, treatment-naïve patients (437 males, 163 females; mean age 61 years; range, 30 to 84 years) with primary or metastatic non-small cell lung cancer who underwent epidermal growth factor receptor mutation testing using Sanger sequencing method were retrospectively analyzed. Archival records and hematoxylin-eosine and immunohistochemically stained sections were re-examined. The thyroid transcription factor-1 and napsin A immunohistochemical stains were performed on tissue array blocks.

Results: Seventy-five mutations were detected in 70 patients (14%). The success rate of testing and intact deoxyribonucleic acid fragment length were significantly higher in the cytological material, compared to tissue specimens ($p<0.001$). The mutation rate in adenocarcinomas was 33.9% for women and 9.4% for men. The most common mutation was L746-E750del in exon 19 (29.3%), followed by the L858R mutation in exon 21 (28%). The mutation rate was the highest in micropapillary (40%) and lowest in solid (5.4%) adenocarcinomas. All epidermal growth factor receptor mutations, except for one, were positive for the thyroid transcription factor-1. The single nucleotide polymorphism Q787Q in exon 20 was observed in 79.6% of patients.

Conclusion: The frequency and distribution of epidermal growth factor receptor mutations in the Turkish patients with non-small cell lung cancer are similar to the European populations. These results also demonstrate that cytological materials are highly reliable for epidermal growth factor receptor mutation testing, and the probability of detection of wild-type epidermal growth factor receptor is low in cases of thyroid transcription factor-1 negativity.

Keywords: Epidermal growth factor receptor, napsin A, non-small cell lung cancer, thyroid transcription factor-1, Turkish population.

ÖZ

Amaç: Bu çalışmada küçük hücreli dışı akciğer kanserli Türk topolumunda epidermal büyüme faktör reseptör mutasyonlarının sıklığı, dağılımı ve morfolojik/immünhistokimyasal özellikleri araştırıldı ve materyal tipi ve mutasyon analizinin teknik başarısı arasındaki muhtemel ilişki incelendi.

Çalışma planı: Eylül 2012 - Aralık 2015 tarihleri arasında Sanger sekanslama yöntemi ile epidermal büyüme faktör reseptör mutasyon testi yapılan, daha önce tedavi edilmemiş, primer veya metastatik küçük hücreli dışı akciğer kanserli toplam 499 ardışık hasta (437 erkek, 163 kadın; ort. yaş 61 yıl; dağılım, 30-84 yıl) retrospektif olarak incelendi. Arşiv kayıtları ve hematoksilen-eozin ve immünohistokimya boyalı kesitler yeniden değerlendirildi. Doku array bloklarında tiroid transkripsiyon faktör-1 ve napsin A immünohistokimyasal boyamaları yapıldı.

Bulgular: Yetmiş hastada (%14) 75 mutasyon saptandı. Sitolojik materyallerde analizin başarı oranı ve intakt deoksiribonükleik asit parça uzunluğu, doku materyallerine kıyasla, anlamlı olarak daha yüksekti ($p<0.001$). Kadınlarda mutasyon oranı %33.9 ve erkeklerde %9.4 idi. En sık görülen mutasyon ekzon 19'da L746-E750del (%29.3) olup, bunu ekzon 21'de L858R (%28) mutasyonu izledi. Mutasyon oranı en yüksek mikropapillerde (%40) ve en düşük solid adenokarsinomalarda (%5.4) izlendi. Biri hariç, epidermal büyüme faktör reseptör mutasyonlarının tümünde tiroid transkripsiyon faktör-1 pozitifliği mevcut idi. Hastaların %79.6'sında ekzon 20'de tekli nükleotid polimorfizmi (Q787Q) izlendi.

Sonuç: Küçük hücreli dışı akciğer kanserli Türk hastalarda epidermal büyüme faktör reseptör mutasyonlarının görülme sıklığı ve dağılımı Avrupa toplumları ile benzerdir. Ayrıca bu sonuçlar, epidermal büyüme faktör reseptör mutasyon analizi için sitolojik materyallerin son derece güvenilir olduğunu göstermekle birlikte, tiroid transkripsiyon faktör-1 negatif olgularda mutasyona rastlama ihtimali çok düşüktür.

Anahtar sözcükler: Epidermal büyüme faktör reseptörü, napsin A, küçük hücreli dışı akciğer kanseri, tiroid transkripsiyon faktör-1, Türk toplumu.

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Lung carcinoma is the leading cause of cancer deaths worldwide.[1,2] The majority of lung carcinomas are non-small cell lung carcinomas (NSCLC), and adenocarcinoma is the most frequent type.[3,4] An important proportion of NSCLC cases is diagnosed at advanced stage and a candidate for chemotherapy and/or personalized therapies.[3,5] The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are important tools in the treatment of NSCLC patients whose tumors show mutations in the EGFR gene.[1,5] Therefore, molecular testing for EGFR mutation has become an essential part of pathological examination in the last decade. The EGFR mutation is more frequent in female and non-smoker patients.[3,6] The mutation rate also shows variation between different ethnic groups, being higher in Asians[1,2,7] and lower in Europeans.[3,5,8] Although there are some reports regarding EGFR mutations from Turkey, most of these are smaller series, and targeted methods are used in larger series.[4,9-18] As a result, little is known about the exact profile of EGFR mutations in the Turkish NSCLC patients.

Thyroid transcription factor-1 (TTF-1), a transcription factor involved in normal thyroid and lung development, plays an active role in sustaining lung cancer and has been proved to be a highly specific and sensitive immunohistochemical marker for lung adenocarcinomas.[19] The TTF-1 expression has been shown to be associated with female gender, never-smoking status, and presence of EGFR mutations.[19,20] Napsin A is an aspartic protease presenting in the epithelial cells of the lung and is expressed in type 2 pneumocytes and also in alveolar macrophages.[19] Napsin A staining is associated with TTF-1 expression and presence of EGFR mutations.[19,20]

In the present study, we aimed to investigate the frequency and distribution of EGFR mutations in the Turkish population with NSCLC and to identify relationship between the TTF-1 and napsin A staining and EGFR mutations.

PATIENTS AND METHODS

This single-center, retrospective study was conducted at Ankara University Faculty of Medicine between September 2012 and December 2015. A total of 600 consecutive, treatment-naïve patients (437 males, 163 females; mean age 61 years; range, 30 to 84 years) with primary or metastatic NSCLC who underwent EGFR mutation testing using the Sanger sequencing method in our pathology lab, irrespective of the tumor stage, were retrospectively analyzed. The specimens were obtained by resection (n=212), biopsy (n=196), and cytology (n=192). Of the tumors, 397 were primary and 203 were metastatic tumors. The primary source of the material was one of the two hospitals of our university in 409 patients and specimens were consultation materials sent for EGFR mutation analysis in the remaining 191 patients.

All hematoxylin and eosin (H-E) stained slides of 600 patients were retrieved from archives and reclassified according to the 2015 World Health Organization (WHO) classification of lung tumors. Carcinomas with adequate tumor tissues were reevaluated for the tumor patterns (i.e., acinar, lepidic, papillary, micropapillary or solid) using a cut-off value of 5%.

A written informed consent was obtained from each patient. The study protocol was approved by the Ankara University Medical School Human Research Ethics Committee (20th April 2015). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Immunohistochemistry

The TTF-1 and/or napsin A stained slides were reviewed, when available. Tissue array blocks were prepared for 57 cases, which were not evaluated for TTF-1 and/or napsin A at the time of the initial diagnosis. Four-micrometer-thick sections were stained for TTF-1 (8G7G3/1, Thermo Fisher Scientific Inc., Waltham, MA, USA) and napsin A (TMU-AD2, Biocare Medical, Pacheco, CA, USA) by streptavidin-biotin peroxidase method using the UltraView Universal DAB Detection Kit on a Ventana (BenchMark XT, Woonsocket, RI, USA) stainer and evaluated on the light microscope (Olympus BX50, Olympus Inc., Tokyo, Japan). For stained cases for TTF-1 and/or napsin A at the time of the initial diagnosis (TTF-1 n=320; napsin A n=293), immunohistochemically stained sections were retrieved and reevaluated with the same criteria. In consultation cases, whose slides and paraffin blocks were not available for tissue array preparation, pathology reports from the external institutions were examined and the TTF-1 and/or napsin A staining results were recorded, if available (TTF-1 n=100; napsin A n=53).

Sequencing

The EGFR mutations in exons 18, 19, 20, and 21 were detected by polymerase chain reaction-based direct Sanger sequencing. Deoxyribonucleic acid (DNA) was extracted from the formalin-fixed, paraffin-embedded (FFPE) tissue obtained from resection or biopsy specimens. For cytological materials, air dried and May Grünwald Giemsa (MGG) stained slides were preferred, when cellularity was adequate; however,

Table 1. Sequences of the primers for EGFR exon 18, 19, 20, and 21

Exon	Forward primer	Reverse primer
18	5'-gctgaggtgacccttgtctc-3'	5'-acagcttgcaaggactctgg-3'
19	5'-gctggtaacatccacccaga-3'	5'-gagaaaaggtgggcctgag-3'
20	5'-catgtgcccctccttctg-3'	5'-gatcctggctccttatctcc -3'
21	5'-cctcacagcagggtcttctc-3'	5'-cctggtgtcaggaaaatgct-3'

EGFR: Epidermal growth factor receptor.

sections from the FFPE cell blocks were also used, where applicable. The DNA extraction was performed using a commercial kit (QIAamp DNA FFPE tissue kit, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Sequencing was performed on an automated single-capillary genetic analyzer (ABI 310; Applied Biosystems, Foster City, CA, USA) with forward and reverse primers (Table 1) separately, and final nucleotide changes were detected by comparing the sequence with the National Center for Biotechnology Information database (reference sequence: NM_005228.2).

Statistical analysis

Statistical analysis was performed using the SPSS for Windows version 15.0 software (SPSS Inc., Chicago, IL, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max), or number and frequency. The EGFR status and clinicopathologic features were analyzed using the chi-square or Fisher's exact test. Pearson correlation analysis was performed to analyze possible correlations between variables. A p value of <0.05 was considered statistically significant.

Table 2. Clinical characteristics and results of EGFR mutation testing (n=499)

	EGFR wild type (n=429)			EGFR mutated (n=70)			
	n	%	Mean±SD	n	%	Mean±SD	p
Age (year)			62.2±10.6			60.6±10.3	>0.05
Gender							<0.001
Female	98	69.5		43	30.5		
Male	331	92.5		27	7.5		
Histological diagnosis							=0.001
Adenocarcinoma	320	82.9		66	17.1		
NSCLC-NOS	78	95.1		4	4.9		
Others	31	100		0	0		
Material type							>0.05
Biopsy	128	86.5		20	13.5		
Resection	143	84.6		26	15.4		
Cytology	158	86.8		24	13.2		
Site							>0.05
Primary	276	84.4		51	15.6		
Metastatic	152	88.9		19	11.1		
Histological subtype							=0.029
Lepidic	16	64		9	36		
Acinar	64	82.1		14	17.9		
Papillary	4	80		1	20		
Micropapillary	3	60		2	40		
Solid	35	94.6		2	5.4		

EGFR: Epidermal growth factor receptor; SD: Standard deviation; NSCLC-NOS: Non-small cell lung carcinoma-not otherwise specified.

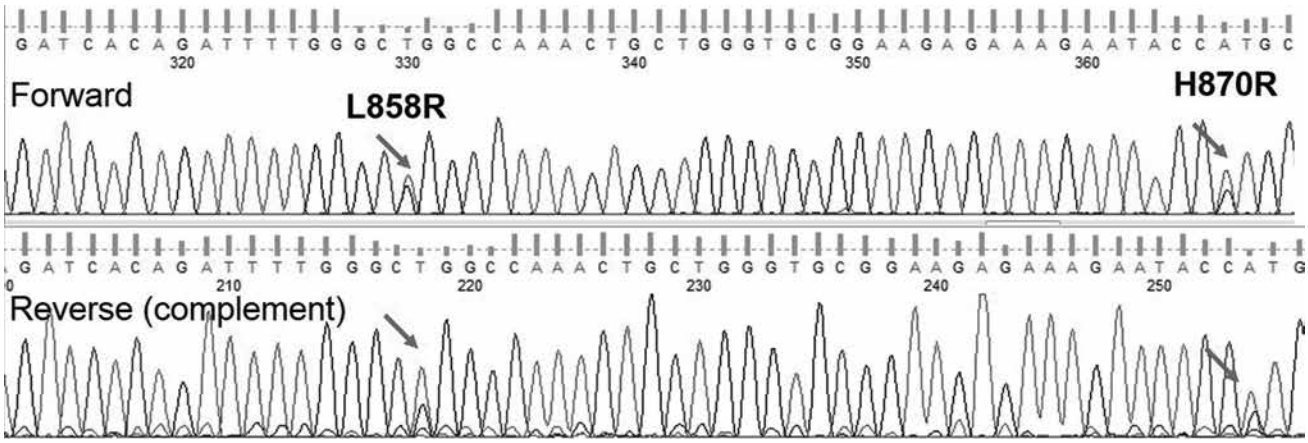

Figure 1. DNA sequencing electropherogram of a doublet mutation (p.H870R+p.L858R) in exon 21 of EGFR gene.
DNA: Deoxyribonucleic acid; EGFR: Epidermal growth factor receptor.

RESULTS

EGFR mutations

Of a total of 600 patients included in the study, the EGFR mutation testing was successful in 499 patients. Clinical characteristics and the outcome of EGFR mutation analysis of successful cases are shown in Table 2. In 495 cases, analyses of all four exons were successful. In additional four cases, an EGFR mutation was detected in one exon, while the analysis of one or more of the remaining exons were unsuccessful. The overall mutation rate was 14% in this series, and of 141 women, 43 (30.5%) showed EGFR mutation, which was more frequent than men (27/358, 7.5%) ($p<0.001$). The EGFR mutations were more frequent in adenocarcinomas (33.9% in women, 9.4% in men), compared to NSCLC-not otherwise specified cases ($p=0.001$). In 67 cases, either DNA extraction was unsuccessful or we were unable to obtain any amplicon. Mutation analysis was able to be performed for only one, two, and three exons; in one, seven, and 30 cases, respectively (n=4 among 38 cases who showed EGFR mutation in one exon and were considered successful per protocol).

Regarding the specimen type, cytological specimens showed the best DNA quality, median DNA fragment length being 600 bp, 400 bp, and 400 bp in cytological, resection and biopsy specimens, respectively ($p<0.001$). Analytical success was higher in cytological specimens ($p<0.001$); 94.8% of cytological specimens, 77.8% of resectional specimens, and 75.5% of biopsy specimens were examined for all four exons. However, analytical success was poor in decalcified specimens ($p<0.001$) and in consultation cases ($p=0.02$).

In this series, a total of 75 mutations were detected in 70 of 499 cases (14%); 5 mutations in exon 18 (6.7%), 40 mutations in exon 19 (53.3%), seven mutations in exon 20 (9.3%), and 23 mutations in exon 21 (30.7%). Of 75 mutations including double mutations in five patients are documented in Table 3 and Figure 1. In addition, 79.6% of our cases showed heterozygous or homozygous single nucleotide polymorphism (SNP) Q787Q in exon 20.

In 179 cases, a predominant adenocarcinoma pattern was evaluated. The most frequent primary pattern was acinar pattern (51.4%), followed by solid (22.9%), lepidic (17.9%), papillary (4.5%), and micropapillary (3.4%) patterns. The EGFR mutations were the most frequently detected in micropapillary adenocarcinomas and rare in solid adenocarcinomas, indicating a significant correlation between the EGFR mutation status and dominant pattern ($p=0.029$). In 11 of 14 (78.6%) EGFR-mutant acinar adenocarcinomas, mutation was located on exon 19; however, it did not reach statistical significance.

TTF-1 and napsin A

The TTF-1 and napsin A expressions were detected in 477 and 403 cases, respectively. In 75.7% and 72.5% of the cases, TTF-1 and napsin A were positive, respectively. Among 57 EGFR-mutant cases, whose TTF-1 expression was also detected, 56 (98.2%) were positive and only one case was negative for TTF-1. Among 48 EGFR-mutant cases, whose napsin A expression was detected, 46 (95.8%) were positive and only two cases were negative for napsin A. The TTF1 positivity and napsin A staining were strongly correlated with the presence of EGFR mutations ($p<0.001$).

Table 3. Genomic alterations in tyrosine kinase domain (exons 18-21) of EGFR gene

Exon	Mutation type	Amino acid change	Diagnosis	Gender	No (%)
18	Missense	p.G719S	ADC	F	1 (1.4)
		p.P699S	ADC	M	
19	Deletion/	p.E746_A750	22ADC	12F/10M	22 (31.6)
	insertion	p.E747_S752	3ADC; 1NSCLC-NOS	2F/2M	4 (5.8)
		p.E747_T751	2ADC	1F/1M	2 (2.9)
		p.E747_P753	2ADC	2F	2 (2.9)
		p.E746_T751>VAins	2ADC	1F/1M	2 (2.9)
		p.K745_E749	ADC	F	1 (1.4)
		p.E746_T751insI	ADC	F	1 (1.4)
		p.L747_A750 insP	ADC	F	1 (1.4)
		p.E746_P753>VSins	ADC	M	1 (1.4)
		p.S752_I759	ADC	F	1 (1.4)
		p.K745_E750	ADC	F	1 (1.4)
		p.746_753insATAT	ADC	M	1 (1.4)
		p.T751_A755insAT	ADC	F	1 (1.4)
20	Missense	p.S768I	NSCLC-NOS	M	1 (1.4)
	Deletion/	p.H773_774 insH	ADC	M	1 (1.4)
	insertion/	p.V769_D770insASV	ADC	F	1 (1.4)
	duplication	p.D770_N771insAWT	ADC	F	1 (1.4)
		p.S768_D770 dupSVD	ADC	F	1 (1.4)
21	Missense	p.L858R	17ADC 1NSCLC-NOS	13F/5M	18 (25.9)
Doublets					
18+18	Missense	p.E709A+p.G719A	ADC	M	1 (1.4)
18+20		p.G719C+p.S768I	ADC	M	1 (1.4)
20+21		p.T790M+p.L858R	NSCLC-NOS	F	1 (1.4)
21+21		p.H870R+p.L858R (Figure 1)	ADC	F	1 (1.4)
21+21		p.V834L+p.L858R	ADC	M	1 (1.4)

A: Alanine; ADC: Adenocarcinoma; D: Aspartic acid; E: Glutamic acid; G: Glycine; H: Histidine; I: Isoleucine; K: Lysine; L: Leucine; N: Asparagine; NSCLC-NOS: Non-small cell lung carcinoma-Not otherwise specified; P: Proline; R: Arginine; S: Serine; T: Threonine; V: Valine; EGFR: Epidermal growth factor receptor.

DISCUSSION

The role of EGFR in carcinogenesis clarified in the 1980s and the first EGFR-TKIs were synthesized in the 1990s. The EGFR somatic mutation frequencies differ among ethnic groups and geographic regions, and Asians show the highest EGFR mutation prevalence worldwide ranging from 33.7 to 59%,[1,2,7] followed by Latin Americans (26%),[21] and it is the lowest in Europeans (5.4 to 15%).[3,5,8]

Our study represents the largest, single-center experience on EGFR mutation status of the Turkish NSCLC patients as assessed by a screening method. We found EGFR mutation in 14% of our patients, which is consistent with the results of other studies from Europe.[8] The results of studies on the Turkish NSCLC patients show a great variation and ranges from 4 to 48.1%.[4,9-18] The number of cases in the series reporting the highest (48.1%, 37.5%, and 44%, respectively) and lowest (4%, 7.1%, 8%, respectively) frequency of EGFR mutations varies between 40 and 52.[9,10,15] Larger series from Turkey including 122, 218, 300, and 959 cases showed an EGFR mutation rate of 14.39%, 28.9%, 15%, and 16.7%, respectively.[4,13,17,18] This discrepancy may be related with small sample size and different methods used in these studies. Even in some of these studies, different methods were used for EGFR mutation testing within the same study,[4,17] and all of four exons were not examined in some others.[9,14,15] In our study, the Sanger sequencing method, which has a low sensitivity, was used, and tumor concentration increased by dissection of tumor from slides. As a result, we believe that 14% EGFR mutation frequency represents the Turkish NSCLC patients successfully. We also believe that using a screening method, which investigate both known and

unknown mutations, is more useful than the targeted methods to detect all mutations present in a newly studied population similar to ours. However, this rate can be expected to increase with a more sensitive analysis method. Similar to previous studies, the most common EGFR mutations were short, in-frame deletions in exon 19, and L858R point mutation in exon 21.[1,3,7] The mutations observed in exon 19 are ranked first in terms of the frequency of EGFR mutations and are reported to be associated with a good response to EGFR-TKIs.[1,7,8] Among all EGFR mutations, the exon 19 mutation rate was found to be 31.55 to 67.8% in European[3,5,8] and 39.4 to 48% in Asian populations.[1,2,7] In this study, EGFR mutations in exon 19 represented 53.3% of all EGFR mutations, and 55% of exon 19 mutations were p.E746_A750del, similar to a previous study.[3] In our study, we detected very rare mutational events in exon 19 (p.746_753insATAT and p.T751_A755insAT), which has not been reported in the literature to date.

Exon 21 mutations are the second most common type of mutations among EGFR mutant cases, p.L858R being the most frequent.[1,5,18] Accordingly, the frequency of exon 21 mutations was 30.7% in this study, while the exon 21 mutation rate was 27.04 to 46.4% in European[3,5,8] and 43 to 49.8% in Asian cohorts.[1,2,7]

Mutations observed in exon 20 are mainly observed as insertion or point mutations and associated with de novo resistance to EGFR-TKIs.[1] The exon 20 mutation rate was reported to be 1.29 to 10.3% in European[3,5,8] and 3.4 to 9.3% in Asian cohorts.[1,2,7] In our study, we observed that EGFR mutations in exon 20 constituted 9.3% of all EGFR mutations.

Mutations in exon 18 are relatively rare and most are point mutations.[7] Frequency of mutations in exon 18 are reported to be 1.29 to 3.2% in European[3,5,8] and 3 to 6.5% in Asian populations.[1,2,7] In our study, EGFR mutations in exon 18 represented 6.7% of all EGFR mutations, and we identified five patients (7.1%) with complex mutations (p.T790M+p.L858R, p.L858R+p.H870R, p.E709A+p.G719A, p.L858R+p.V834L, p.G719C+p.S768I).

In previous studies investigating the relationship between the dominant adenocarcinoma pattern and EGFR mutations, solid adenocarcinomas were mostly associated with the wild-type EGFR.[6,22] Similarly, the lowest EGFR mutation rate was observed in solid adenocarcinomas in the present study. In addition, micropapillary adenocarcinomas showed the highest incidence of EGFR mutations consistent with some previous reports.[6,22]

On the other hand, there is a limited number of studies evaluating the relationship between the mutation types and dominant pattern. As shown in a previous study, reporting exon 19 mutations were more common in acinar adenocarcinomas, and 11 of 14 cases (78.6%) showed mutations in exon 19 with an acinar pattern.[22] This rate is of relevance, when compared to 53.3% overall frequency of exon 19 mutations in this series. Despite this, it did not reach statistical significance ($p>0.05$). However, further studies with larger series may be useful to reveal a significant relationship.

In previous studies, TTF-1 positivity was observed in 89.4 to 99% of EGFR-mutant tumors, whereas TTF-1 was negative in only 1 to 10.6% of cases.[4,20] Our study results showed a significant correlation between the EGFR mutational status and TTF-1 protein expression, indicating that patients with TTF-1-negative adenocarcinomas had at least a 99% chance of being wild-type EGFR. Although TTF-1 status should not be used to include or exclude cases for EGFR testing, it may be used for prioritizing molecular tests in resource-poor settings or when the available tissue is not adequate for all molecular tests.

Lee et al.[19] and Jie-Liu et al.[20] reported that napsin A positivity rate was 94.6%, and 72.3%, respectively in EGFR mutant cases. Our study results demonstrated a significant association between napsin A expression and EGFR mutation, consistent with the results of Lee et al[19] and Jie et al.[20]

Furthermore, in our study we observed a high frequency (79.6%) of SNP Q787Q in the EGFR gene which is more common in Caucasians (23 to 28.3%)[23] than in Asians (70.6 to 82.7%).[24,25] This SNP frequency was 79% in the EGFR-mutant cases and 80% in wild-type cases, probably due to ethnic origin, rather than a disease-related phenomenon.

In the present study, we also showed that cytological materials were highly reliable for EGFR mutation testing. Cytological specimens showed a higher DNA fragment length, compared to surgical and biopsy specimens, showing a higher DNA quality, which can be attributed to the lack of damaging effect of formalin fixation on DNA. Analytical success rate was also higher in cytological specimens.

The main limitation of the study is the low sensitivity of Sanger sequencing. Variants with low allele frequency were unable to be detected in the FFPE blocks or cytological specimens which had limited tumor cells (>20%).

In conclusion, the frequency and distribution of epidermal growth factor receptor mutations in the Turkish patients with non-small cell lung cancer are similar to the European populations. These results also demonstrate that cytological materials are highly reliable for epidermal growth factor receptor mutation testing, and thyroid transcription factor-1 negativity appears to be a good predictor of wild-type mutations. Further large-scale, prospective studies are needed to gain a better understanding of this issue.

Declaration of conflicting interests

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