Increased EGFR mRNA Expression Levels in Non-Small Cell Lung Cancer

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Cite this article as: *Tasdemir S, Taheri S, Akalin H, Kontas O, Onal O, Ozkul Y. Increased EGFR mRNA Expression Levels in Non-Small Cell Lung Cancer. Eurasian J Med 2019; 51(2): 177-85.*

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Received: October 04, 2015
Accepted: October 26, 2015
Available Online Date: January 25, 2017

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DOI 10.5152/eurasianjmed.2016.0237

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ABSTRACT

Objective: In this study, we investigated the frequency of Epidermal growth factor receptor (EGFR) gene mutations, the level of EGFR mRNA and protein expressions in Turkish population for indicating substantial differences in the frequency of EGFR mutations, EGFR amplification and EGFR protein expression between populations and the effect of these parameters in response to EGFR tyrosine kinase inhibitors.

Materials and Methods: The study included 34 patients with non-small cell lung cancers. The RNA and DNA were extracted from the normal and tumor side of the lung tissue removed by surgery. To investigate the most common mutations in the EGFR gene, exon 19 was sequenced and mutation specific PCR was performed for detecting the L858R mutation in exon 21. EGFR mRNA expression was measured by relative quantitative reverse transcription PCR. The EGFR protein levels were detected with immunohistochemistry methods from the sections of the patients' paraffin blocks.

Results: No EGFR mutation in exon 19 or L858R mutation in exon 21 were detected in the patients. Overexpression of EGFR gene mRNA was identified in 16 of 34 (%47) patients and overexpression of EGFR protein was detected in 15 of 34 (%44) patients. Statistical analysis was not significant for the correlation between sex, age, smoking, histopathology, pathological stage and overexpression of EGFR mRNA and protein.

Conclusion: It was found that in Turkish population, EGFR mutation in exon 19 and L858R mutation were very rare, EGFR protein expression was similar and EGFR mRNA expression significantly increased compared to the literature. Markedly increased EGFR mRNA expression ratios in the absence of activating mutations showed that identifying the EGFR mRNA expression level for prediction of response to EGFR tyrosine kinase inhibitors might be significant in the Turkish population.

Keywords: NSCLC, EGFR, mutation, mRNA, protein, Turkey

Introduction

Lung cancer is one of the most common cancers in humans and is the most common cause of death from cancer in the world. Non-small cell lung cancer (NSCLC) constitutes about 85% of lung cancers and it is divided into subgroups as squamous cell carcinoma, adenocarcinoma, large cell carcinoma and others [1]. The treatment of lung cancer is planned mainly according to the stage of the cancer, the patient's performance status and comorbid diseases. A large proportion of patients with NSCLC are diagnosed at stage 3 and stage 4, and chemotherapy is usually recommended as the first-line treatment option [2]. Chemotherapy is very toxic, especially for the elderly and patients with poor performance. Therefore, in recent years there are efforts for developing targeted molecular-based drugs.

Epidermal growth factor receptor (EGFR) is a critical oncogenic factor involved in the occurrence and progress of NSCLC. EGFR is overexpressed in the majority of patients with NSCLC and is an important target in the treatment. EGFR is a member of the EGF-related tyrosine kinase receptor family [3]. There are two specific EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, which are enhanced and used in clinic for the treatment of advanced NSCLC. An objective response rate is about 10% for unselected NSCLC patients. Female patients, non-smokers, East Asians and patients with adenocarcinoma have a much higher response rate [4]. Today, molecular markers, which affect the response to EGFR-TKIs, have also been identified.

Many somatic mutations were identified in the EGFR gene in NSCLC. Most of the mutations are localized in the tyrosine kinase domain (exons 18-21) of the EGFR gene. The amino acids 746-753 encoded by exon 19 and the amino acid 858 encoded by exon 21, which comprise more than 80% of all detected mutations, are two mutation hotspots. Sensitivity to EGFR-TKIs has been identified in small frameshift deletions of exon 19 and arginine for leucine substitution at 858th amino acid of exon 21 (L858R) [5-8]. This is the most important molecular mechanism in lung cancer. The seven phase-2 study made with gefitinib or erlotinib showed that response to TKIs are more than 87% and life expectancy is between 7.7 to 14 months without progression in patients with EGFR mutation positive NSCLC [9]. This period is much longer than the time that is provided by chemotherapy in unselected patient population and other target therapies (4-6 months). Gene amplification is a mechanism responsible for the overexpression of oncogenes. Increased EGFR copies have been identified in about 30% of NSCLC patients with FISH analysis and this is often associated with poor prognosis [10]. Increased EGFR copy is an effective indicator for better treatment response to EGFR-TKIs [5,7]. High EGFR copy number is often associated with EGFR somatic mutations [5,8]. EGFR mutation rates in American and European patients (10%) are too low when compared to Asian patients (30-50%). Still, a response to EGFR-TKI therapy is received in significant proportion of patients without the EGFR mutation and increased EGFR copy number might be the cause. Japanese patients with EGFR gene amplification do not benefit from gefitinib treatment and there is no known reason for this [11].

EGFR protein expression is very high (40-80%) in patients with NSCLC and is associated with poor prognosis [12]. Both positive [7,13] and a negative relationship [14] is found in the literature between the levels of EGFR protein and EGFR TKIs sensitivity. The EGFR protein level is usually associated with EGFR gene copy number [7,10]. Both IHC and FISH positive patients can benefit from EGFR-TKIs therapy.

There are significant differences in the prevalence of EGFR gene mutations in patients with lung cancer from different ethnic groups. The frequency of these mutations are 1-10% in the US and European patients [6, 15], 19-26% in Southeast Asians patients [16], and about 58% in East Asian women [17]. The studies showed that the incidence of EGFR mutations in Middle Eastern society was similar to western society. While the amplification of the EGFR gene was seen about 30% in East Asian lung cancer patients, it is below 10% in the US and Australian patients. The frequency of EGFR amplification is higher in the Middle Eastern society (16%) than the western population (6-9%) [10].

In this study EGFR mutation frequency and EGFR mRNA and protein expression levels were investigated in Turkish population due to different EGFR mutation, amplification and protein expression rates between communities and their effects in response to the tyrosine kinase inhibitors. This study may determine the molecular predictors in predicting the efficacy of tyrosine kinase inhibitors in the NSCLC patients in Turkish population.

Materials and Methods

Patients

The study was ethically approved by the local ethics committee of Erciyes University (accession number: 2008/10) in accordance with the ethical standards of Helsinki Declaration and supported by Erciyes University Scientific Research Department with TST-08-399 project number. Written informed consent for study tissue DNA, RNA and protein was obtained from each patient in the study. Clinical data were obtained from patients' medical records. The following criteria were used to classify smoking status: never smokers were defined as those with lifetime exposure of 100 cigarettes or less; former smoker, who had stopped smoking at least 12 months before diagnosis and current smoker, who had stopped smoking 1 to 12 months before diagnosis or current smoking.

Primary cancer tissue and normal tissue samples were obtained from 34 NSCLC patients who had undergone lobectomy at the Erciyes University Department of Thoracic Surgery. RNA was extracted from fresh tissue samples as soon as possible. Tissue samples were collected at -80 C for DNA isolation and isolated together.

L858R (exon 21) mutation analysis by mutation specific PCR

Genomic DNA was isolated using Genelute mammalian genomic DNA miniprep kit (Sigma-Aldrich) according to manufacturer's guidelines. The EGFR gene exon 21 was amplified by PCR. Two specific primer sets were designed for the wild type sequence and the L858R mutation in exon 21 by introducing a wild type or mutated nucleotide at the 3′ terminal end (Table 1). A 20 ul PCR reaction mixture contained 5 ul of 10 PCR buffer, 50 nmol of $MgCl_2$, 10 pmol each of sense and antisense primer, 6 nmol of dNTPs, 2 U of Taq polymerase and 100 ng of sample DNA. PCR was performed on Rotorgene Real-Time PCR System (Qiagen, Germany) under the following conditions: 95°C for 10 min, followed by 35 cycles of 94°C for 30s, 64°C for 30s, 72°C for 30s and final extension at 72°C for 5 min. PCR products were electrophoresed in % 2 agarose gels and visualized under UV.

EGFR exon 19 mutation analysis by direct sequencing

Sample DNA was amplified by PCR using the primers indicated in Table 1. A 20 ul PCR reaction mixture contained 5 ul of 10 PCR buffer, 50 nmol of $MgCl_2$, 10 pmol each of sense and antisense primer, 6 nmol of dNTPs, 2U of Taq polymerase and 100 ng of sample DNA. PCR was performed on Rotorgene Real-Time PCR System (Qiagen, Germany) under the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 64°C for 45 s, 72°C for 30 s and final extension at 72°C for 10 min. DNA products producing a positive band on agarose gel were used for further steps. Amplified DNA was purified using Genomic DNA purification kit (Fermantas). 10 ng of PCR products was applied for the sequencing reaction using a Dye terminator cycle sequencing quick start kit (Beckman Coulter). EGFR exon 19 sequence analysis was performed using CEQTM 8000 Genetic Analysis System (Beckman Coulter). The reference coding sequence of EGFR was obtained from the NCBI (NCBI Reference Sequence: NG_007726.1) [18].

EGFR mRNA expression by relative quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using UltraClean tissue RNA isolation kit (MO BIO) following the manufacturer's protocol. The cDNA was transcripted by using Transcriptor first strand cDNA synthesis kit with random hexamers (Roche; Germany). We prepared four PCR tubes for each patient to quantify the expression of EGFR-GAPDH in tumor samples and EGFR-GAPDH in normal samples. PCR reactions in a final volume of 25 µl: 2 µl cDNA, 18 µl distilled water, 1 ul $MgCl_2$, 2 µl LightCycler faststart DNA master SYBR green I mix (Roche Diagnostics) and 2 µl primers for each EGFR and GAPDH gene (Table 1).

For each cDNA sample, expression levels of EGFR and the reference gene (GAPDH) were analyzed using the Rotorgene Real-Time PCR System (Qiagen, Germany). Cycle conditions of the relative qRT-PCR were preincubated at 95°C for 10 min, followed by 45 amplification cycles of 95°C for 8 s, 62°C for 15 s, 72°C for 10 s, and a melting curve analysis, which ran

Table 1. Primers for exon 21 PCR, exon 19 sequence analysis and expression of EGFR gene

	Sense	Antisense
Exon 21-Wild type	5′-gcttggtgcaccgcgacctg-3′	5′-cgcacccagcagtttggcgA-3′
Exon 21-L858R	5′-gcttggtgcaccgcgacctg-3′	5′-cgcacccagcagtttggcgC -3′
Exon 19	5’-gcaatatcagccttaggtgcggctc-3’	5’-catagaaagtgaacatttaggatgtg-3’
EGFR	5’-ctcagccacccatatgtacc-3’	5’-ggtctcgggccattttgg-3’
GAPDH	5’-acaacagcctcaagatcatcag-3’	5’-tcttctgggtggcagtgatg-3’

Melt (65-95°c), at 95°C hold 0 sec, at 65°C hold 15 secs and at 95°C hold 0 sec. qRT-PCR analysis and calculation of quantification cycle (Cq) values for relative quantification were performed by the Rotorgene Real-Time PCR software (Qiagen, Germany).

The gene expression of EGFR gene was normalized with GAPDH. The relative quantification of EGFR gene was calculated using the following formula [19] and the expression ratio greater than one is considered as overexpression (positive).

$$\text{Expression ratio} = \frac{\text{TEGFR/TGAPDH}}{\text{NEGFR/NGAPDH}}$$

TEGFR: measured expression of EGFR gene in tumor sample
TGAPDH: measured expression of the housekeeping gene, GAPDH in tumor sample
NEGFR: measured expression of EGFR gene in normal sample
NGAPDH: measured expression of the housekeeping gene, GAPDH in normal sample

EGFR protein expression by immunohistochemistry

Formalin-fixed paraffin embedded cancer tissue sections were used for the study. The immunohistochemical study was performed for protein expression and graded using Rabbit anti-human EGFR monoclonal (Clone SP9) antibody at a dilution of 1/100 (Spring Bioscience) according to the manufacturer's instructions. The slides were counterstained with hematoxylin.

IHC analysis was independently reviewed by two pathologists who were blinded to the clinical outcome data, and differences in interpretation were resolved by consensus. EGFR expression was scored based on the intensity and percentage of IHC staining cells. The staining pattern was cytoplasmic and membranous. The intensity score was defined as follows: Score 0, no staining; score +1, faint staining; score +2 moderate staining (nearly at the same staining level as observed in the normal bronchial epithelium); score3, strong staining. The total score was calculated by multiplying the intensity score and the percentage of tumor cells showing characteristic staining (0-100%) producing a total range of 0-300. For statistical analyses, scores of 1-100, 101-200, and 201-300 were considered grade 1, grade 2 and grade 3, respectively. Samples that exhibited grade 3 immunostaining scored as overexpression (positive).

Statistical analysis

Statistical analyses were performed with SPSS software, version 15.0. Comparisons of EGFR mRNA expression and protein expression level within clinical patient characteristics were performed by using the Fisher exact test or the x^2 test. A p value equal or less than 0.05 was considered statistically significant. EGFR mRNA expression and protein expression were compared by kappa, using the Landis and Koch criteria.

Results

Patients

The sex, age, histopathology, pathologic stage distribution, and smoking status of the cases that diagnosed as non-small cell lung cancer with histopathological examination are listed in detail in Table 2.

L858R (exon 21) mutation assed by mutation specific PCR

The L858R mutation was detected in none of the patients.

EGFR exon 19 mutations assed by direct sequencing

The mutations in exon 19 of EGFR gene was detected in none of the patients.

EGFR mRNA expression assessed by relative qRT-PCR

Patients' EGFR expression ratios (TEGFR/TGAPDH / NEGFR/NGAPDH) are shown in Table 3. EGFR expression ratios of the 16 patients are bigger than one.

EGFR protein expression assed by immunohistochemistry

Examples of the staining intensity values of +1, +2, +3 used for evaluation of the measurement of EGFR protein expression in patients are shown in Figure 1. Fifteen samples that exhibited grade 3 immunostaining scored as overexpression are shown in Table 3.

Table 2. Clinical patient characteristics

	n	%
Sex		
Male	31	91
Female	3	9
Age		
<39	2	6
40-49	5	15
50-59	11	32
60-69	8	23.5
>70	8	23.5
Smoking		
Never smoker	3*	9
Former smoker	11	32
Current smoker	20	59
Histology		
Adenocarcinoma	10	29
Squamous	23[a]	67
Large cell	1	3
Pathologic stage		
IA-IB	16	47
IIA-IIB	12	35
IIIA-IIB	6	18
Total	34	100

*; two patients have tandoori story >20 years
[a]; one patient has squamous + small cell carcinoma histology
Never smoker: who had smoked fewer than 100 cigarettes in their lifetime. Former smoker: who had stopped smoking at least 12 months before diagnosis. Current smoker: who had stopped smoking 1 to 12 months before diagnosis or current smoking

Among 34 patients that exhibited, 16 (47%) had EGFR gene mRNA overexpression, 15 (44%) had EGFR protein overexpression. The statistical results of EGFR gene mRNA expression and EGFR protein expression according to clinical patient characteristics are shown in Table 4. According to the statistical results, there was no significant difference between EGFR mRNA and protein expression with clinical characteristics.

The level of agreement for EGFR protein expression determined by immunohistochemical analysis and EGFR mRNA expression determined by qPCR demonstrate a $\mathbf{K}$ of 0.348 (Table 5). A $\mathbf{K}$ of 0.348 indicates a fair level of agreement according to Landis and Koch criteria (values < 0 as indicating no

Table 3. Clinical patient characteristics and study results

Case	Sex	Smoking	Age	Histology	Pathologic stage	Expression ratio	IHCG
1	M	Former smoker	54	Squamous	IA	0.90	3
2	F	Never smoker*	50	Squamous	IIB	0.03	2
3	M	Current smoker	38	Adenocarcinoma	IB	0.76	3
4	M	Current smoker	55	Adenocarcinoma	IIIA	10.26	2
5	M	Former smoker	66	Squamous	IIB	4.45	3
6	M	Current smoker	48	Adenocarcinoma	IB	0.28	2
7	M	Current smoker	75	Squamous	IB	0.04	1
8	M	Former smoker	55	Adenocarcinoma	IIA	0.05	2
9	M	Current smoker	47	Squamous	IIIA	0.08	2
10	M	Former smoker	66	Squamous	IIB	0.28	2
11	F	Never smoker*	72	Adenocarcinoma	IB	1.39	3
12	M	Current smoker	44	Adenocarcinoma	IIB	1.19	2
13	M	Current smoker	63	Squamous	IIA	0.64	2
14	M	Former smoker	75	Squamous	IIIB	0.11	3
15	M	Current smoker	51	Squamous	IIIA	0.04	2
16	M	Current smoker	54	Squamous	IIA	2.13	1
17	M	Current smoker	57	Squamous	IIIA	0.49	2
18	M	Current smoker	49	Squamous	IB	64.10	3
19	M	Current smoker	74	Squamous	IB	13.29	2
20	M	Current smoker	40	Squamous	IB	0.31	1
21	M	Current smoker	56	Squamous	IB	20.42	3
22	M	Former smoker	70	Squamous	IB	1.42	3
23	M	Former smoker	58	Adenocarcinoma	IB	0.25	2
24	M	Former smoker	73	Squamous	IIB	1.37	3
25	M	Current smoker	61	Squamous	IIIA	0.08	1
26	M	Current smoker	60	Adenocarcinoma	IIB	2.49	3
27	M	Current smoker	58	Squamous	IB	15.31	3
28	M	Former smoker	68	Squamous	IIA	1.01	2
29	M	Former smoker	73	Squamous	IA	1.93	3
30	M	Current smoker	61	Large cell	IB	0.83	3
31	M	Current smoker	50	Adenocarcinoma	IIB	8.71	3
32	F	Never smoker	38	Adenocarcinoma	IB	0.02	2
33	M	Former smoker	68	Squamous	IIB	0.28	3
34	M	Current smoker	70	Squamous[a]	IA	17.00	2

*; have tandoori story >20 years
[a] ; Squamous + small cell
IHCG: Immunohistochemical grading, M: Male, F: Female
Expression ratio = TEGFR/TGAPDH / NEGFR/NGAPDH

agreement, 0–0.20 as slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1 as almost perfect agreement) [20].

Discussion

There are significant differences in the prevalence of EGFR gene mutations in patients with lung cancer from different ethnic groups. The studies showed that the incidence of EGFR mutations in Middle Eastern society was similar to western society. The frequency of EGFR amplification is higher in the Middle Eastern society than the western population [6,10,15,17]. EGFR gene mutation, amplification and expression studies in the literature in NSCLC patients and our study are shown in Table 6.

In the majority of publications, EGFR mutations were identified by sequence and mutation specific PCR + gel electrophoresis; gene amplification by FISH and qPCR and EGFR protein expression was evaluated by IHC method. EGFR protein expression positivity (with IHC method) varies between 40-80% of NSCLC tumors as shown in Table 6. Different results may be because of different detection methods used in measuring EGFR pro-

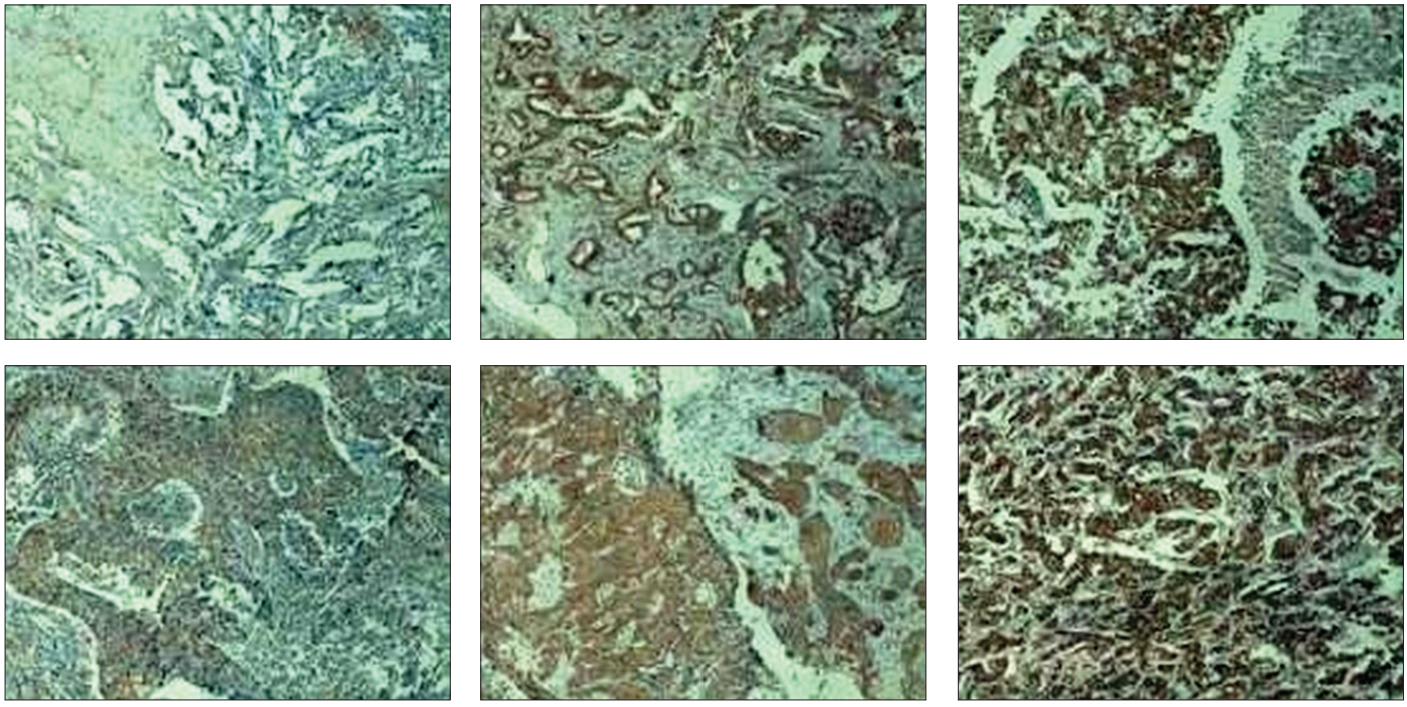


Figure 1. Representative immunohistochemical staining intensity (left to right +1, +2, +3) for EGFR. Upper row: Adenocarcinoma, lower row: Squamous carcinoma

Table 4. Characteristics of clinical patient according to EGFR mRNA and EGFR protein expression

	EGFR mRNA expression			EGFR protein expression		
Clinical characteristics	negative	positive	p	negative	positive	p
Sex						
Male	16 (52)	15 (48)	0.55	17 (55)	14 (45)	0.59
Female	2 (67)	1 (33)		2 (67)	1 (33)	
Smoking						
No	2 (67)	1 (33)	0.86	2 (67)	1 (33)	0.28
Yes	16 (52)	15 (48)		17 (55)	14 (45)	
Histology						
Squamous	12 (52)	11 (48)	0.52	13 (56.5)	10 (43.5)	0.42
Adenocarcinoma	5 (50)	5 (50)		6 (60)	4 (40)	
Large cell	1	0		0	1	
Pathologic stage						
IA-IB	8 (50)	8 (50)	0.21	7 (44)	9 (56)	0.22
IIA-IIB	5 (42)	7 (58)		7 (58)	5 (42)	
IIIA-IIB	5 (83)	1 (17)		5 (83)	1 (17)	
Age						
Average	56	62	0.1	56.5	61.5	0.19

Table 5. Correlation between expression level of EGFR protein and mRNA

	EGFR mRNA expression		
EGFR protein expression	Negative	Positive	Total
Negative	13	5	18
Positive	6	10	16 (%47)
Total	19	15 (%44)	34

tein. Different antibodies, different scoring systems and different protocols were used in different laboratories. In this study the EGFR mRNA and protein expression were measured by qRT-PCR and IHC methods, respectively.

In this study for determining mRNA expression, the EGFR expression in tumor tissue was compared with normal tissue in the same patient (TEGFR/TGAPDH/ NEGFR/ NGAPDH). This method led to the identification of the exact expression results for each patient because EGFR expression normally seen in non-tumor tissue of the patients were excluded (background expression). The differences between initial mRNA amounts of different patients are balanced with the usage of GAPDH as an internal control. This analysis method was used in the study of Brabender et al. [21] for the evaluation of EGFR and HER2-neu mRNA expression in patients with NSCLC, Mafune et al. [22] for expression evaluation in squamous cell esophagus carcinomas and Bong et al. [23] for identifying the expression of colorectal carcinoma.

The frequency of EGFR molecular pathologies change among societies. Evaluation of the usage of molecular pathology targeted drugs in different societies or development of molecular pathology targeted drugs are needed. In our study, EGFR gene changes, which are common in the etiology of NSCLC, are planned to be investigated in the NSCLC patients of Turkish society for predicting the effectiveness of TKIs and for determining the molecular changes that can be used for the response to EGFR therapy. In our study, about 80% of EGFR gene muta-

Table 6. Summary of selected reports on EGFR gene, protein study in NSCLC

				EGFR gene mutation	EGFR gene amplification		EGFR protein expression		EGFR mRNA expression
Author	Year	Country	Case	Mutation ratio (%)	FISH (%)	qPCR (%)	IHK (%)	Western Blot (%)	qRT-PCR (%)
Paez [6]	2004	US	119	13					
Hirsh [38]	2005	US	82		32				
Dacic [35]	2006	US	199		9		16		
Hirsh [10]	2003	US	352		32				
Bell [39]	2005	US	453			7			
Shigematsu [16]	2005	US /Australia	158	8					
Brabender [21]	2001	US/Germany	83						34
Marchetti [15]	2005	Italy	860	5					
Cortez [40]	2005	Spain	83	12					
Cappuzzo [7]	2005	US-Europe	89	17	32		59		
Dziadziuszko [34]	2006	US-Europe	82			51*			22
Taron [8]	2005	US/Europe/Asia	28	35	32				?
Tsao [13]	2005	Canada	325	23	45		57		
Pinter [41]	2008	Hungary	126	13	40		59		
Qin [42]	2005	China	41	24					
Liang [43]	2012	China	120			38		34	
Shigematsu [16]	2005	East Asia	361	30					
Sasaki [44]	2006	Japan	575	20					
Suziki [45]	2005	Japan	130	28			56		
Yokoyoma [46]	2006	Japan	349	29					
Sonobe [31]	2007	Japan	53	32			79		?
Takano [5]	2005	Japan	66	59		44			
Suziki [47]	2005	Japan	181		23		34		
Huang [48]	2004	Taiwan	101	39					
Han [49]	2005	Korea	90	19					
Ahn [32]	2008	Korea	92	28		41	72		
Al-Kuraya [30]	2006	Saudi Arabia	47	3	15		70		
Seyhan [36]	2010	Turkey	98				52		
Cetin [29]	2010	Turkey		4 (n=2)				46	
Gorgisen [24]	2013	Turkey	16	0 (n=0)					
Unal [25]	2013	Turkey	48	43 (n=18)					
Akca [26]	2013	Turkey	52	48 (n=25)					
Bircan [27]	2014	Turkey	25	44 (n=11)					
Dogan [28]	2014	Turkey	42	7 (n=2)					
Gundogdu [37]	2014	Turkey	26				54		
This study	2015	Turkey	34	0 (n=0)			47		44

*: The average of the results obtained and specified percentage of those, which indicated greater than the average; ?: Percent was not given; qPCR: quantitative PCR; qRT-PCR: quantitative reverse transcription PCR

tions in the literature were checked and no mutation detected.

The reason for this negativity may be the number of our patients, only three female patients, and three non-smoker patients, two of whom have tandoori story more than 20 years. With this study, the average mutation rate of the Turkish population (267 case, 58 cases have mutated) is 22% between 0-48% [24-29]. The frequencies of these mutations are 1-10% in America and Europe [6, 15]. EGFR mutations are rare in Middle Eastern patients, similar to the rates in the Western society (%3). Significantly increased EGFR amplification rates (15%) have shown the need to determine the changes of gene copy number in response to anti-EGFR therapy in the Middle East patients [30].

Increased EGFR mRNA and protein expression levels were stated in patients with EGFR mutations when compared to patients without gene mutations [31]. Many publications have shown that EGFR mutations are associated with increased EGFR gene copy number and EGFR expression. EGFR gene mutations and increased amplification were found to be the best parameter in determining clinical progression in the Korean patients treated with erlotinib [32] and in Japanese patients treated with gefitinib [5]. In addition, researchers have suggested investigating the effects of these molecular markers in different ethnic groups. Similar assessment could not be done in our study due to absence of mutations.

Amador et al. found that head and neck cancer cells with increased EGFR mRNA expression were more susceptible to erlotinib [33]. Taro et al. studied with 28 advanced NSCLC patients and detected increased EGFR mRNA expression levels in patients with EGFR mutations when compared to patients without mutations, but it was statistically insignificant [8]. EGFR mRNA expression was shown to be a major biomarker in response to gefitinib and progression free survival [34]. The percentage of mRNA expression positivity in NSCLC patients was indicated as 22-34 % [21, 34]. Our study shows 44% positivity, which is higher than in the literature. Significantly increased EGFR mRNA expression rates (44%) suggest that mRNA expression changes should be addressed in determining the response to anti-EGFR therapy in the Turkish community.

EGFR mRNA expression by qRT-PCR, EGFR gene dosage by qPCR, and EGFR copy number by FISH in patients with gefitinib treated non-small cell lung cancer were analyzed in order to determine the association with treatment outcome, clinical, and biological features [34]. EGFR mRNA expression was higher in responders to gefitinib compared to non-responders. EGFR mRNA expression was higher in FISH-positive patients and in patients with positive EGFR immunostaining, but not in patients with EGFR mutations. EGFR gene dosage did not predict response to gefitinib and was not associated with EGFR mutation status, FISH positivity, mRNA expression and EGFR protein expression [34].

In the literature, Dacic et al., reported gene amplification correlated with protein expression, and it seems that gene amplification is a mechanism for protein overexpression in a subset of squamous cell carcinomas of the lung [35]. Although Hirsch et al. [10] found EGFR protein overexpression in all tumors with gene amplification, Dacic et al. demonstrated that protein expression does not necessarily require gene amplification, suggesting that other mechanisms such as gene mutation and transcriptional or posttranscriptional factors might have a role.

In our study, no correlation was found between clinic-pathological characteristics of patients and the expression levels of mRNA and protein. EGFR mRNA and protein overexpression were detected in 44% and 47% of patients, respectively. The agreement between EGFR mRNA and protein expression indicated a fair level (k = 0.348). EGFR protein overexpression was found in 67% of patients who increased mRNA expression. Although the remaining 33% of the patients increased EGFR mRNA expression, the increase in the protein expression was not detected. The reasons for this may be grading differences in the absence of objective evaluation criteria in IHC method, tumor cell contamination in the normal tissue or normal cell contamination in the tumor tissue. mRNA can also be impaired or translation can be blocked due to post-transcriptional events such as miRNA mechanisms.

In this study, even though approximately 85% of the mutations indicated in the literature were studied, no mutation detected. The mutation rates in European and Middle Eastern societies are about 1-10% and lower than East Asia community [6, 15]. The EGFR protein expression is between 16-80% in other communities and 46-54% [29, 36, 37] in Turkish society; we found a ratio of 47% in our study consistent with the literature. EGFR gene amplification has been identified in many different rates such as 9-45% by FISH and 7-51% by qPCR. EGFR mRNA expression was found 22-34% in the literature, which is lower than our positive rate of 44%. Based on the hypothesis that in general, increased gene amplification is associated with increased gene expression, our positive mRNA expression rates are higher than amplification rates measured by FISH and qPCR and higher when compared to European society, but shows compliance with East Asian societies.

In conclusion, markedly increased EGFR mRNA expression ratios in the absence of activating mutations show that identifying an increase in the EGFR mRNA expressions for the prediction of response to EGFR tyrosine kinase inhibitors may be significant in Turkish population. Further studies of patients treated with EGFR inhibitors would be necessary to estimate the influence of EGFR mRNA expression on the response to therapy in Turkish population.

Ethics Committee Approval: The study was ethically approved by the local ethics committee of Erciyes University (accession number: 2008/10) in accordance with the ethical standards of Helsinki Declaration.

Informed Consent: Informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - S.T. Design - S.T., Y.O.; Supervision - Y.O.; Materials - O.O., O.K.; Data Collection and/or Processing - S.T.; Analysis and/or Interpretation - S.T., S.T., H.A.; Literature Review - S.T; Writing - S.T; Critical Review - S.T., Y.O.;

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The study was supported by Erciyes University Scientific Research Department with TST-08-399 project number.

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