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Original Article

Evaluation of Epidermal Growth Factor Receptor Gene Mutations in an Iranian Population with Non-Small Cell Lung Carcinoma

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Abstract

Background: Mutations of the epidermal growth factor receptor (*EGFR*) gene, predominantly in exons 18-21, have been highlighted to function as the crucial predictors of the response rate of patients with non-small cell lung cancer (NSCLC) to *EGFR* tyrosine kinase inhibitors (TKIs).

Methods: This study was performed at Tehran University of Medical Sciences. Data and information were retrospectively collected from the period between Dec 2010 and Apr 2014. Exons 18 to 21 of the *EGFR* were analyzed for any potential mutation by PCR, accompanied by DNA sequencing on 160 with pathological confirmation of NSCLC.

Results: Demographically, the male to female ratio was approximately 2:1, and a substantial difference in age between sexes was not observed (P=0.065), but a noticeable difference was found in the smoking variable, where 77.8% of males were smokers compared to 17.3% of women (odds ratio (OR) (95% CI) = 16.72 (7.15-39.11)). We found a frequency of 10.63% (17/160) for mutations found in exons 19 and 21, nonetheless, no mutations in exon 18 and exon 20 were observed. The most frequently observed mutations were c.2235_2249, del and c.2240_2257, del in exon 19 and p. L858R in exon 21. The c.2253A>G was found as a novel mutation that was the rarest mutation detected in this work. Interestingly, a remarkable negative association was revealed between smoking and mutation rates in NSCLC patients (OR (95% CI) = 0.13 (0.04- 0.46).

Conclusion: The occurrence of *EGFR* mutations is largely varied among the different states of Iran, probably due to variations in ethnicity, smoking rate, and sex ratio of participants.

Keywords: Non-small cell lung cancer (NSCLC); Epidermal growth factor receptor (*EGFR*); Gene Mutation; Clinicopathological data; Iran

Introduction

Lung cancer is the second most prevalent cancer and the leading cause of cancer deaths in men and women in the US based on the American cancer society, with an estimated 228,820 new

cases (112,520 in women and 116,300 in men) and approximately 135,720 deaths (1, 2). Lung cancer incidents are increasing by 38% to 2.89% million cases by 2030 (3). The most common form of lung cancer is NSCLC, which accounts for 84% of all diagnosed lung cancers with varying survival rates that are largely dependent on the stage of the disease (SEER) (4). While there are many therapeutic approaches to the treatment of patients with NSCLC, they remain almost marginally successful and, due to poorly understood pathological processes, NSCLC cases have proven to be difficult to treat (4, 5).

Increased understanding of cell signaling pathways involved in cell survival of NSCLC cells led to identification of genetic aberrations that influence all aspects of tumorigenesis, applied as the critical targets for personalized/targeted therapy (6, 7). The epidermal growth factor receptor (*EGFR*) that act as a transmembrane receptor tyrosine kinase, is expressed in some normal tissues and has been illustrated to be tremendously upregulated or hyper-activated in NSCLC cells. *EGFR*-targeted therapies have been disclosed to improve remarkably progression-free and overall survival in a subset of NSCLC patients carrying "activating" mutations in the *EGFR* gene (8).

A previous investigation reported overexpression/hyperactivation of the *EGFR* gene in 25%-89% of NSCLC cases, which frequently result from various types of mutations that occurred in exons 18, 19, and 21 or whole gene amplification (8). These mutations can lead to cell proliferation or anti-apoptosis activities occurred due to the constitutive activation of signal transduction pathways, irrespective of the existence of extracellular ligands. Around 10% of advanced NSCLC patients respond to TKIs targeting *EGFR*. Retrospective studies have shown that 75% of TKI-responsive tumors carry an activating mutation. NSCLC patients carrying these mutations have been shown to have different responses to TKIs, such as erlotinib (Tarceva) and gefitinib (Iressa), which may be due to variations in ethnicity, smoking rate, and sex ratio of participants (8, 9).

Therefore, we aimed to evaluate the sequences of exons 18, 19, and 21 in *EGFR* to find out the frequency of divers' mutations in NSCLC patients in an Iranian population that required a targeted therapy.

Materials and Methods

Subjects

DNA samples were taken from tumor tissues of one hundred and sixty NSCLC patients referred to the different Oncosurgery centers, including Masih Daneshvari Hospital (Tehran, Iran) and Al-Zahra Hospital (Isfahan, Iran). Via review of medical records and questionnaires, clinicopathological characteristics, demographic data, history, and treatment information of subjects were collected (Table 1).

Table 1: Demographic and clinicopathological characteristics of enrolled patients

Characteristics	*Subgroups*	*Frequency (%)*
Age (year)	Mean (± Std. Deviation)	60.43 (± 12.3)
Gender	Male	108 (67.5)
	Female	52 (32.5)
Smoking (±drugs)	Yes	93 (58.1)
	No	67 (41.9)
TKI-based treatment*	Yes	34 (21.3)
	No	126 (78.8)
Survival (month)	Mean (± Std. Deviation)	14.06 (±11.87)
Survival status	Living	91 (56.9)
	Dead	69 (43.1)

*. Tyrosine kinase inhibitor

Data and information were retrospectively collected from the period between Dec 2010 and Apr 2014 but information of the patients was updated recently. To confirm the adequacy of tissue specimens and the histologic diagnosis of adenocarcinoma, the H&E slides were examined by the pathologist. Formalin-fixed paraffin-embedded (FFPE) tissues obtained from primary tumors and metastatic sites (biopsies or surgically resected specimens) were used for mutation analysis.

The Ethical Committee of Tehran University of Medical Sciences approved this study (IR.TUMS.MEDICINE.REC.1397.640). In addition, all the participants fill and sign the consent form and participated voluntarily in the study.

DNA extraction, Polymerase Chain reaction (PCR), and sequencing

Fifty mg of tumor specimens were used to extraction of genomic DNA by FavorPrep™ Tissue Genomic DNA Extraction Mini Kit, according to the manufacturer's protocol. The quantity and quality of extracted DNA were determined by NanoDropND-1000 Spectrometer (Thermo-Scientific, Boston, MA) and gel electrophoresis, respectively. Genomic sequence of *EGFR* was extracted from www.ensemble.org (10) and specific primers were designed for exons 18, 19, and 21 (Table 2) by utilizing Primer3 online software (http://bioinfo.ut.ee/primer3/) (11) and finally were then blasted against the human genome using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (12). PCR were performed in a 25 µl reaction mixture in a thermal cycler instrument (Applied Biosystems, GeneAmp 2720, Singapore) under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 sec, annealing for 30 sec at 65 °C for exons 18 and 20, as well as 64 °C for exons 19 and 21, extension at 72 °C for 30 sec and a final extension at 72 °C for 7 min. For the identification of mutations, the amplified PCR product was exposed to direct nucleotide sequencing.

Table 2: The sequence of primers used in this study

Genes	*Primer type*	*Primer sequence*
Exon 18	F	CAA GTG CCG TGT CCT GGC ACC CAA GC
	R	CCA AAC ACT CAG TGA AAC AAA GAG
Exon 19	F	CAGCAATATCAGCCTTAGGTGC
	R	AGCAGGGTCTAGAGCAGAGCAG
Exon 20	F	CCATGAGTACGTATTTTGAAACTC
	R	CATATCCCCATGGCAAACTCTTGC
Exon 21	F	CTA ACG TTC GCC AGC CAT AAG TCC
	R	GCT GCG AGC TCA CCC AGA ATG TCT GG

F: forward primer, R: reverse primer

Statistical Analysis

SPSS ver. 19 (IBM Corp., Armonk, NY, USA) was used to statistical analyses. The differences between groups for quantitative and categorical variables were assessed by Student's t-test and Chi-Squared (χ^2) test, respectively. All the experiments were two-sided and $P<0.05$ were considered statistically significant.

Results

Baseline characteristics of the subjects

Table 1 presents the demographic and clinicopathological variables in 160 NSCLC patients enrolled in the study with mean age (± standard deviation (±SD)) of 60.43 (± 12.3), that all were diagnosed in the late stage of the disease (stage IV). One hundred eight cases out of 160 cases of NSCLC were men (67.5%). Besides, 93 cases

(58.1%) of participants smoked/consumed (±drugs) during their lifetime. Regarding the survival duration and status, the mean survival time (month) (Mean ± SD) was 14.06 ±11.87, and 91 cases (56.9%) were alive by the time of enrollment.

The distribution of age, survival duration, and smoking (±drugs) between male and female patients were shown in Table 3. The mean age (± SD) of men and women patients was 61.62 (± 12.21) and 57.96 (± 11.36), respectively (*P*=0.065) (Table 3. a). Furthermore, the survival duration (± SD) for men and women subjects was 14.51 (± 11.80) and 13.12 (± 12.09), respectively (*P*=0.493) (Table 3. a). We found that 77.8% of men smoked (±drugged), compared to 17.3% of women (odds ratio (OR) (95% CI)=16.72 (7.15-39.11); *P*<0.001) (Table 3. b). Despite no significant difference between smoking (± consuming drugs) and survival duration in all participants and women patients (*P*=0.058 and *P*=0.883, respectively), a noticeable difference was observed in male patients (*P*=0.012) (Table 3. c). Table 3d presents the remarkable association between smoking (± consuming drugs) and survival status in all patients. Furthermore, 54.8% of patients with a positive history of smoking died, while the mortality rate in patients with no history of smoking was 26.9% (OR (95% CI)=3.30 (1.67- 6.67); *P*=0.001).

Table 3: Distribution of age, survival duration, and smoking (±drugs) between male and female patients, as well as the association of smoking with survival duration/ status

a. Potential differences between male and female patients in age and survival duration variables					
Variables	Subgroup	N	Mean	Std. Deviation	Sig. (2-tailed)
Age	Male	108	61.62	12.21	0.065
	Female	52	57.96	11.36	
Survival	Male	108	14.51	11.80	0.493
	Female	52	13.12	12.09	
b. Association between gender and smoking (±drugs) among patients					
Gender	Smokers (%)	No. smokers (%)	OR (95 % CI)	Sig. (2- sided)	
Male	84 (77.8)	24 (22.2)	**16.72 (7.15-**	**< 0.001***	
Female	9 (17.3)	43 (82.7)	**39.11)**		
c.)Potential differences between smoking and survival duration in all participants, as well as in male and female patients					
All patients					
Variable	subgroups	N	Mean	Std. Deviation	Sig (2-tailed)
Smoking	Yes	93	15.54	12.37	0.058
(±drugs)	No	67	12.00	10.91	
Males					
Variable	subgroups	N	Mean	Std. Deviation	Sig (2-tailed)
Smoking	Yes	89	10.32	6.21	**0.012***
(±drugs)	No	19	15.40	12.52	
Females					
Variable	subgroups	N	Mean	Std. Deviation	Sig (2-tailed)
Smoking	Yes	4	13.19	10.53	0.883
(±drugs)	No	48	13.19	12.30	
d. Association between smoking and survival status in all patients					
Smoking (±drugs)	Living (%)	Dead (%)	OR (95% CI)	Sig. (2- sided)	
Yes	42 (45.2)	51 (54.8)	**3.30 (1.67- 6.67)**	**0.001***	
No	49 (73.1)	18 (26.9)			

* Bolded- *P* values < 0.05 were considered statistically significant

EGFR mutations

In the current study, we examined exons 18-21 of the *EGFR* gene to detect any mutations in 160 Iranian patients with NSCLC, which all were diagnosed with stage IV. Although no mutations were found in exons 18 and 20, different types of mutations were detected in exons 19 and 21 (Table 4.a) (Fig. 1).

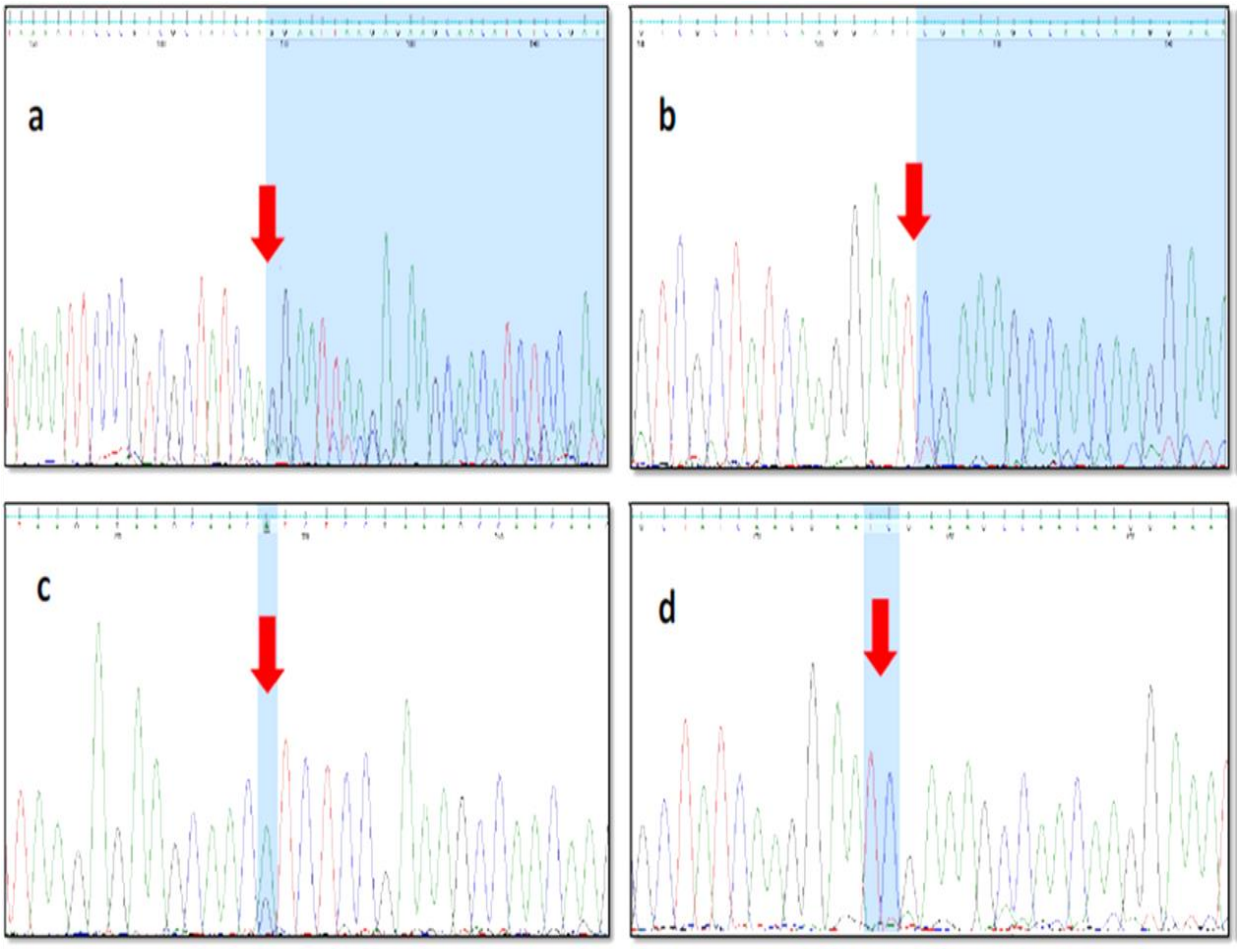

Fig. 1: Sequence chromatograms of the *EGFR* gene mutations located in the exons 19 and 21, including (a) heterozygous for c.2235_2249delGGAATTAAGAGAAGC, (b) heterozygous for c.2240_2257delTAAGAGAAGCAACATCTC, (c) heterozygous for c.2253A>G, and (d) homozygous for c.2240_2257del TAAGAGAAGCAACATCTC. Arrows represents the sequence change

Regarding the exon 19, we found three mutations, including: 1) c.2235_2249 deletion (Del) (GGAATTAAGAGAAGC) in 10 patients (6.5%), 2) c.2240_2257 Del TAAGAGAAGCAACATCTC in four patients (2.5%), and 3) c.2253A > G in one patient (0.6%). Moreover, we found only one mutation in exon 21 of NSCLC patients known as c.2573T > G (p. L858R) observed in two patients (1.3%). According to Table 4.b, a slight increase of mutation frequency in studied exons of *EGFR* was found in women with NSCLC against men patients, however, it was not statistically significant (P=0.097). An interesting result in our study was the significant negative association between smoking and mutation rates in studied exons of *EGFR* gene in NSCLC patients (OR (95% CI)=0.13 (0.04- 0.46); P=0.002) (Table 4.c).

Table 4: Frequency of *EGFR* mutations, as well as their associations with gender and smoking variables

a. Mutation frequency in three studied exons, including)18, 19, and 21				
E18- Mutation types	Frequency	Percent	Valid percent	Cumulative percent
No mutation	160	100.0	100.0	100.0
E19- Mutation types	Frequency	Percent	Valid percent	Cumulative percent
No mutation	145	90.6	90.6	90.6
c.2240_2257 del TAAGAGAAGCAACATCTC	4	2.5	2.5	93.1
c.2235_2249 del GGAATTAAGAGAAGC	10	6.3	6.3	99.4
c.2253A>G	1	0.6	0.6	100.0
E21- Mutation types	Frequency	percent	Valid percent	Cumulative percent
No mutation	158	98.7	98.7	98.7
c.2573T>G, p.L858R	2	1.3	1.3	100.0
b. Distribution of *EGFR* mutation between male and female patients				
Gender	Mutated (%)	No- mutated (%)	OR (95% CI)	Sig. (2- sided)
Male	8 (7.4)	100 (92.6)	2.61 (0.95-	0.097
Female	9 (17.3)	43 (82.7)	7.24)	
c. Association between smoking and *EGFR* mutation rate				
Smoking (±drugs)	Mutated (%)	No- mutated (%)	OR (95% CI)	Sig. (2- sided)
Yes	3 (3.2)	90 (96.8)	**0.13 (0.04-**	**0.002***
No	14 (20.9)	53 (79.1)	**0.46)**	
* Bolded- *P* values < 0.05 were considered statistically significant				

Discussion

Lung cancer, except non-melanoma skin cancer, is the fourth most common cancer in Iran and the second leading cause of cancer mortality following stomach malignancy (13). Most NSCLC patients have remote metastases or pleural effusion at the time of initial diagnosis and, owing to the poor efficacy of anticancer agents, are not candidates for surgical care or even systemic chemotherapy. Accordingly, targeted drugs are most often used for NSCLC cases, either alone or in combination with chemotherapy. One of the most interesting genes targeted by these drugs is *EGFR* expressed in most patients with NSCLC and functions as a critical oncogene in different aspects of NSLC tumorigenesis, including proliferation, angiogenesis, metastasis, inhibition of apoptosis, and chemoresistance (14). To date, available therapies targeting *EGFR* can be categorized into small-molecule *EGFR* TKIs and a monoclonal anti-EGFR antibody become the standard preferred treatment for patients with advanced NSCLC harboring *EGFR* mutation, which strongly affects the success of these therapies (14). Accordingly, screening the mutations of *EGFR* are currently being used as important predictors of clinical response to EGFR-TKIs (15). Furthermore, the frequencies and nature of driver mutations in NSCLC cases have been shown to be largely distinct, primarily based on ethnicity (16). Regarding Iran, as a highly multi-ethnic/racial country, there are several reports on the incidence of *EGFR* mutations in NSCLC patients (17, 18). Therefore, evaluating the frequencies of *EGFR* mutations in different ethnicities in this country is essential.

In the current work, the mutations' frequency detected in the *EGFR* gene was 10.63%, that all were found in exons 19 and 21, while no mutations were observed in exons 18 and 20. Furthermore, we found that mutations in exon 19 accounted for 88.4% (15/17), while mutations found in exon 21 accounted for 11.6% (2/17) of all the mutations. Different incidences of *EGFR*

mutations in NSCLC have been recorded around the world probably due to racial differences, including 12% in Oceania, 15% in Europe, 21% in Africa, 22% in North America, 26% in the Indian subregions, 36% in South America, and 40% in East Asia (16, 19-22). Consistently, *EGFR* mutations entirely were occurred only in exons 19 and 21 in Iranian patients with lung adenocarcinoma, with frequencies of 60% in exon 21 and 40% in exon 19 (17). An in-frame deletion (c.2235-2249 del) in exon 19, which comprised up to 58.8% of all observed mutations, was the most common mutation in our sample, accompanied by another in-frame deletion in the same exon (c.2240-2257 del) with a 23.5% frequency and a missense mutation p. L858R (c.2573T > G) in exon 21 with a frequency of 11.8%. The substitution mutation of c.2253A > G was the rarest found in the present work, which accounted for up to 5.8% of all detected mutations. Similar to our results, an in-frame deletion was reported in exon 19 as the most common *EGFR* mutation comprising up to 71.4% of all mutations in lung adenocarcinoma patients (Shiraz, Iran) (18). Moreover, in our investigation, two main resistant *EGFR*-TKI mutations (exon 20 inserts and T790M) were not found that is to consistent with two previous studies conducted in Iran (17, 18).

Previous investigations pinpointed the importance of gender and smoking status as clinical predictors of *EGFR* mutations in NSCLC subjects. Regarding the gender variable, our results revealed that female patients were more susceptible to the *EGFR* mutations compared to men (17.3% versus 7.4%), however, this difference was no statistically significant (*P*=0.097). This finding is similar to many previous reports in other geographic locations, including Asian populations with observed mutations in 60% of females and 37% of males, in European countries with a frequency of 22% in females and 9% in males, in North America with 28% in females compared to 19% in males, in Africa with 48% in females compared to 8% in males, and in Iran, this frequency was 30% in women and 26.7% in men (16, 20, 21, 23). In the present study, we interestingly observed a higher frequency of *EGFR* mutations in non-smokers compared to smoker patients (20.9% versus 3.2%), meaning a remarkable negative association (protective effect) between smoking and occurring mutations in *EGFR* gene (OR (95% CI) =0.13 (0.04- 0.46) and *P*=0.002). These data were consistent with the Mohammadzadeh group's findings, even though their result was not statistically significant (18). Similar to our findings, a systematic review showed a significantly increased frequency of *EGFR* mutation in non-smokers with NSCLC compared to smoker patients, in which the frequency of assessed mutations was demonstrated to be 64% in non-smokers against 33% in smokers in Asian Pacific region, as well as 35% in non-smokers and 8% in smokers in Europe (16). Interestingly, *EGFR* and K-ras (a key downstream effector of the *EGFR* axis) mutations were mutually exclusive in the *EGFR* signaling pathway. In contrast to the negative association between smoking and *EGFR* mutations; there is a strong positive association between smoking and K-ras mutations (24, 25). Accordingly, the activation of the *EGFR* signaling pathway is mediated by *EGFR* gene activating mutations in non-smokers, while this is mediated by smoking dependent K-ras activating mutations in smoker patients (24, 25).

Ultimately, our findings illustrated higher mortality in smokers NSCLC compared to nonsmoker patients (54.8% versus 26.9%; OR=3.30 (1.67-6.67; *P*=0.001), which is similar to previous works (26-28). Smoking is more associated with mutations in genes associated with a worse prognosis and therefore higher mortality of NSCLC. Non-smoker patients are more likely to have mutations in the *EGFR* gene correlated with a better prognosis due to the achievement of the new-targeted therapies.

EGFR mutations frequency in studied Iranian subpopulation with NSCLC from Fars ethnicity is lower than South and North America, Asia-Pacific, India, and also most European countries, however, higher than Austria and Australia, and almost equal to Finland, Norway, Sweden, Greece, and especially Germany (29-38). The frequency of *EGFR* gene mutations in both female

and male patients with NSCLC is almost the same as the Iranian subpopulation studied in the present work with ~ 7% and ~17% in men and women, respectively.

Conclusion

Analysis of *EGFR* mutation is suggested to be considered as the preferred test for selecting treatment options, and determining the *EGFR* mutation has great usefulness on prognosis and prediction of patient survival. Furthermore, it is suggested to performed mutation detection in populations with different ethnicities, and even in all subpopulations of a country with the overall same ethnicity, but with variable smoking rates due to the influence of smoking rate on the distribution of *EGFR* mutations.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

There is no conflict of interest to declare.

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