DOI:10.31557/APJCB.2022.7.2.125

RESEARCH ARTICLE

Assessing Mutations in Treatment Response-related Genes in Egyptian Patients with Non-small Cell Lung Cancer

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Abstract

Background: Epidermal growth factor receptor (EGFR) and Kirsten rat sarcoma viral oncogene homolog (K-RAS) are the most common driver genes in patients with non-small cell lung cancer adenocarcinomas (NSCLC/ADC), which affects treatment. Therefore, this study determines the frequency and patterns of EGFR and K-RAS mutations in Egyptian patients with NSCLC/ADC and correlates them with clinicopathological features. **Methods:** A retrospective analysis of 139 patients diagnosed with NSCLC/ADC and screened for EGFR and K-RAS mutations was conducted; further evaluating clinicopathological characteristics and mutational status. **Results:** This study included 101 males and 38 females with a median age of 57.7 ± 10.5 years. EGFR mutations were detected in 22.3% (12.2% in exon 19, 8.6% in exon 21, and 1.4% in exon 18) and K-RAS mutations in 17.3% (15.8% in codon 12 and 1.4% in codon 13), whereas combined mutations were detected in nine patients (6.5%). Furthermore, EGFR mutations were non-significantly more common in females and nonsmokers, contradicting K-RAS mutations, which were more common in males and smokers. **Conclusion:** EGFR and K-RAS mutations are common in Egyptian patients with NSCLC/ADC (National Cancer Institute experience). Their incidences were between the Asian Pacific and Europeans. Also, their mutations led to dysregulation in tyrosine kinase activity, which correlates with the late disease stage and poor progression. Therefore, analyzing them should be done to determine a better treatment method and predict survival outcomes.

Keywords: Epidermal growth factor receptor- kirsten rat sarcoma viral oncogene homolog- non-small cell lung cancer

Asian Pac J Cancer Biol, **7 (2)**, 125-132

Submission Date: 03/10/2022 Acceptance Date: 04/21/2022

Introduction

For the past two decades, targeting mutations in the kinase domain of the epidermal growth factor receptor (EGFR) gene has been an important approach used to treat non-small cell lung cancer (NSCLC) tumors. Unfortunately, according to global cancer incidence, mortality, and prevalence (GLOBOCAN) 2020, lung cancer is the leading cause of cancer-related death, with an estimated 1.8 million deaths [1].

The EGFR receptor is a transmembrane glycoprotein receptor, and its gene is located on chromosome 7's short arm (7p11.2) that encodes the 170 kDa Type I transmembrane growth factor receptor with tyrosine kinase (TK) activity [2]. Additionally, EGFR belongs to the human epidermal growth factor receptor/erbB family of receptor TKs, which received great attention due to its strong association with malignant transformation and cellular proliferation. Homodimerization and/or heterodimerization in response to ligand-binding activate the TK, causing autophosphorylation of the receptor's cytoplasmic domain. This allows interaction with other molecules, activating three major downstream signaling pathways. These pathways are important in maintaining and growing normal cells [3-6]. Therefore EGFR is a vital gene responsible for cell activity; its mutation is

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accompanied by a dysregulation in EGFR TK activity, which is closely correlated with the late disease stage and poor progression of NSCLC [7,8]. The most common mutations in the EGFR gene are mostly in the four exons (18–21) that are clustered around the TK domain [9]. They include point mutation G719X (G719A, G719C, G719S) in exon 18, in-frame deletions and in-frame insertions in exon 19, point mutation T790M and insertions in exon 20, and point mutations (L858R and L861Q) in exon 21. However, of these mutations, only two types account for about 85% of the EGFR mutations: deletions in exon 19 and L858R point mutation in exon 21 [10].

In 2004, EGFR mutations were found to be sensitive to targeted therapies called tyrosine kinase inhibitors (TKIs), which were the first molecular changes in NSCLC cases. Also, chimeric monoclonal antibodies (panitumumab and cetuximab) and TKIs (gefitinib, erlotinib, and afatinib) are among these developed strategies that target EGFR [11] [12] They were chosen to compete with ATP (adenosine triphosphate) binding site inhibitors at the active site of EGFR kinase, therefore preventing and blocking vital EGFR pathways [13,14].

The TKIs of the first-generation (erlotinib, gefitinib, and icotinib) induce reversible ATP-binding site blockade and halt the downstream signaling pathway. Almost all patients who received this generation developed resistance. The T790M point mutation was among the most common mutations in patients who developed resistance to first-generation TKIs. Second-generation compounds (afatinib and dacomitinib) formed irreversible covalent bonds with all homo- and hetero-dimers of the ErbB family receptors and blocked the transphosphorylation to inhibit signaling. Thus, having a greater effect to overcome or delay the resistance of first-generation TKIs, but it also acquired resistance. The third and most recent generation (osimertinib, rociletinib, olmutinib, and lazertinib) is a novel treatment due to its ability to bind to the T790M EGFR-mutant receptor. However, patients who received third-generation TKIs developed resistance. Other factors, such as small cell lung cancer transformation and downstream gene mutations [Kristen rat sarcoma viral oncogene homolog (K-RAS) and v-raf murine sarcoma viral oncology homolog B (BRAF)] were discovered in patients who developed resistance [12] [15]. The RAS family proteins are GTPase proteins that switch various cellular activities. This family includes K-RAS, H-RAS, and N-RAS genes [16], and its mutation is usually associated with TKI resistance, as mentioned above. Therefore, coexisting driver mutations in the same tumor affect the therapeutic outcome and survival rate of patients with NSCLC, whether treated by chemotherapy or targeted therapies [17]. Codons 12/13 K-RAS mutations were described in approximately 20% of lung adenocarcinomas and are associated with tobacco consumption [18].

The prevalence of EGFR mutations varied greatly across ethnic backgrounds and geographical locations due to differences in patient demographics, study designs, assays used, number of sequenced exons, tumor source, and eligibility criteria for trial enrolment [19]. In 2015 study, 50 lung tissue biopsies from Egyptian patients were screened for EGFR mutations in which, deletion in exon 19 was the only detected mutation [20]. Recently, in a 2020 study, EGFR mutation was detected in 15/34 (44.1% of the tumors) using EGFR XL Strip Assay kit [21] and in 2022 anther study was published where EGFR mutations were detected in 40.8% of NSCLC Egyptian patients [22].

This study determines the frequency and pattern (s) of EGFR and K-RAS mutations in NSCLC adenocarcinoma (ADC) cases from Egypt. Furthermore, it correlates the detected mutations to the patients' relevant clinicopathological data.

Materials and Methods

Subjects

A retrospective cohort study was conducted in which 139 patients with NSCLC/ADC were recruited from the National Cancer Institute (NCI) data clinics between 2012 and 2015. Ethical approval was granted by the Institutional Review Board of the Egyptian NCI (approval no.2010011003.3). Notably, this study was conducted following the Helsinki Declaration.

Sample preparation and DNA Extraction

From each specimen, a representative hematoxylin and eosin stained slide was prepared and reviewed by a pathologist to ensure the presence of adequate representative tumor cells in the section before extraction. DNA was then extracted and purified from the selected formalin-fixed, paraffin-embedded (FFPE) tissue blocks (each of the five sections is 4–5 micron thick) using the QIAamp DNA FFPE tissue kit (Qiagen, Germany) (catalog number: 56404) according to manufacturers' protocols.

EGFR mutational analysis

EGFR mutations were detected using the Therascreen EGFR RGQ PCR kit (Qiagen, Germany) (catalog number: 870111) on Rotor-Gene Q MDx 5plex HRM instrument (Germany) according to manufacturers' protocol. This ready-to-use kit detects somatic mutations in the EGFR gene by PCR. In addition, it detects actionable somatic mutations against a background of wild-type (WT) genomic DNA using ARMS (Amplified Refractory Mutation System) and Scorpion techniques. The detected mutations were exon 19 deletions, T790M, L858R, L861Q, G719X, and S768I, and three insertions in exon 20. Mutation types are listed in Table 1.

The test was performed twice; the first sample assessed the total amplified DNA, ensuring that each sample contained a sufficient amount of DNA. The second is to determine the presence or absence of EGFR mutations and the type of mutation (s).

K-RAS mutational analysis

According to a method by Hadija (2007) [23], each sample underwent PCR-RFLP (PCR with restriction fragmentation length polymorphism) analysis for PCR amplification of codons 12 and 13 of the K-RAS gene. The PCR products were then digested using restriction

enzymes Bst NI for codon 12 mutations and Bgl I for codon 13 mutations. The test was conducted in four stages: first, PCR and digestion; then second, PCR and digestion. For UV visualization, the second digestion's final codon product was then separated on a 2% agarose gel stained with ethidium bromide. Also, product sizes were estimated by comparing them to the 50 bp DNA ladder. The 157 bp and 128 bp fragments represent mutation at codon 12 and WT, respectively. For codon 13, the 157 bp and 125 bp fragments represent mutation and WT, respectively [23].

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences v.24. Numerical data were summarized using means and standard deviations (SD) or medians and ranges. Categorical variables were summarized as numbers and percentages; differences were analyzed with the X2 (chi square) and Fisher's exact tests. Finally, P values of ≤0.05 were considered significant.

Results

Of the 139 patients enrolled in this study, 101 (72.7%) were males, and 38 (27.3%) were females. The mean age was 57.7 years (26–81). Fifty-two cases (37.4%) were nonsmokers, however, 87 (62.6%) were current and former smokers. All included patients were diagnosed with adenocarcinoma, and most cases were in stage III (26 patients: 19.8%) and stage IV (99 patients: 75.6%). One hundred and twenty-two patients received chemotherapy (92.4%), whereas surgery was performed in 20 patients (15.3%), and radiotherapy (RTH) was given to four patients (3%). The main demographic and clinical data of the patients included in this study are illustrated in Table 2.

Furthermore, EGFR mutations were detected in 31/139 cases (22.3%), and they were non-significantly more frequent in females than males (31.6% vs. 18.8%) and nonsmokers compared with smokers (28.8% vs. 18.4%) (Table 3).

The most common detected mutations were deletion in exon 19 followed by exon 21 point mutation, accounting for 12.2% and 8.6% of all 139 samples, respectively. However, only two cases had a mutation in exon 18 G719X (1.4%) (Figures 1 and 2).

K-RAS mutations were found in 24/139 (17.3%) patients and were non-significantly more frequent in smokers compared with nonsmokers (18.4% vs. 15.4%) and males rather than females (17.8% vs. 15.8%) (Table 3). The most common K-RAS mutations were found in codon 12 (22 patients, 15.8%), whereas codon 13 was found in two patients (1.4%) (Figures 3 and 4).

Nine patients (6.5%) had mutations in both genes (EGFR and K-RAS), whereas 93 (66.9%) were WT for both genes.

Table 1. List of Mutations and COSMIC IDs

Mutation	Exon	Base change	COSMIC ID
T790M	20	2369C>T	6240
L858R	21	2573T>G	6224
L861Q	21	2582T>A	6213
S768I	20	2303G>T	6241
G719A	18	2156G>C	6239
G719S	18	2155G>A	6252
G719C	18	2155G>T	6253
Insertions	20	2307_2308ins9	12376
		2319_2320insCAC	12377
		2310_2311insGGT	12378
Deletion	19	2235_2249del15	6223
		2235_2252>AAT(complex)	13551
		2236_2253del18	12728
		2237_2251del15	12678
		2237_2254del18	12367
		2237_2255>T (complex)	12384
		2236_2250del15	6225
		2238_2255del18	6220
		2238_2248>GC (complex)	12422
		2238_2252>GCA(complex)	12419
		2239_2247del9	6218
		2239_2253del15	6254
		2239_2256del18	6255
		2239_2248TTAAGAGAAGC (complex)	12382
		2239_2258>CA (complex)	12387
		2240_2251del12	6210
		2240_2257del18	12370
		2240_2254del15	12369
		2239_2251>C (complex)	12383

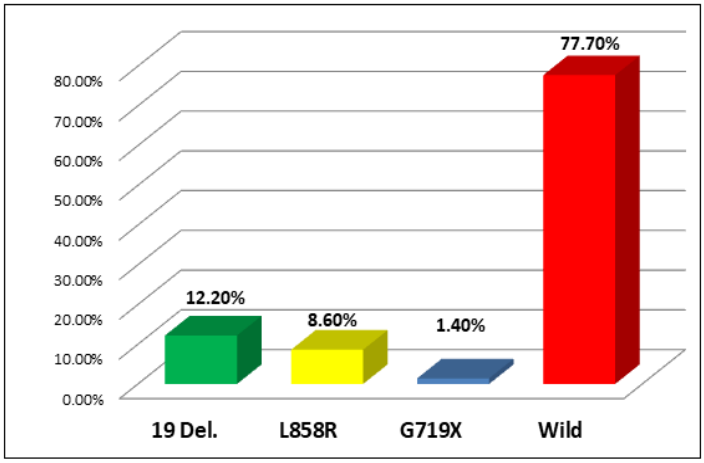

Figure 1. Frequency of EGFR and Its Mutational Pattern

High grades showed a significantly higher proportion of all clinicopathological factors with EGFR and K-RAS mutations (Table 3).

Discussion

This study detected EGFR gene mutations in 31 patients with NSCLC/ADC (22.3%), whereas K-RAS mutations were found in 24 patients (17.3%). Previous studies by Benbrahim (2018) [24] and Tfayli [19] reported that EGFR mutation frequency in patients in the Middle East and Africa is higher than that in white populations but still lower than that reported in Asia. These results agree with those in this study, which showed that the mutational incidence of EGFR was higher than that of the Asian pacific regions but lower than the European incidences [25-27].

Most of the last decade studies showed that Asia-Pacific regions (China, Hong Kong, Japan, Malaysia, Philippines, Korea, Singapore, Taiwan, Thailand, Vietnam, and India) had the highest EGFR gene mutation frequency [25] [28,29] however, K-RAS mutation incidences in these regions were low [30-32].

Conversely, in European regions (Czech Republic, Finland, France, Germany, Greece, Italy, Lithuania, the Netherlands, Norway, Portugal, Russia, Slovakia, Spain, Sweden, Turkey, and United Kingdom), EGFR mutation frequencies were lower than Asia's incidences and those reported in this study [33,34]. Meanwhile, K-RAS mutational frequencies in the European regions were much higher than in the Asian regions [18] [32] [34,35]. Furthermore, in our study, the prevalence of K-RAS mutation was also between the European and Asian regions.

In this study, EGFR and K-RAS combined gene mutations were found in nine patients (6.5%), and this incidence is higher than that observed in a Vietnamese study, in which combined mutations were 1.7% [36].

The reported EGFR mutational incidence in this study was close to a Brazilian study conducted in 2019 where EGFR mutation was found in 22.7% of 444 Brazilian patients, whereas K-RAS mutation was in 20.4% [18]. Also, the incidence in our study agreed with that in a study conducted in Morocco in 2013 [37].

Although the prevalence of EGFR incidence rate in this study agreed with the Middle East region rate (2.9%–28.7%) [20] [24] [37], it was lower than that demonstrated in Iraq (27.53%) [38]. Also, it was lower in the four Gulf regions (United Arab Emirates, Kuwait, Oman, and Qatar) in 2020 (36.9%) [39], but higher than that detected in the Saudi populations, Lebanese populations, and the multicentre study done in Lebanon, Jordan, and Iraq [40-44].

Table 2. Patient's Characteristics

Characteristics		Number (Percentage)
Gender	Male	101 (72)
	Female	38 (27.3)
Age (years)	≤59	69 (49.6)
Mean ± SD (range)	>59	70 (50.4)
	male	58.4±10.5 (30-81)
	female	55.8±10.4 (26-75)
	Total	57.7±10.5 (26-81)
Smoking	nonsmokers	52 (37.4)
	Smokers	87 (62.6)
Tumor size (cm)	mean± SD	6.3±2.7
	range	(1-11)
Performance status	1	98 (76)
	2	24 (18.6)
	3	7 (5.4)
Pathology	adenocarcinoma	139(100)
Tumor grade	1	5 (4.4)
	2	67 (58.8)
	3	42 (36.8)
Tumor stage	I	3 (2.3)
	II	3 (2.3)
	III	26 (19.8)
	IV	99 (75.6)

SD; standard deviation

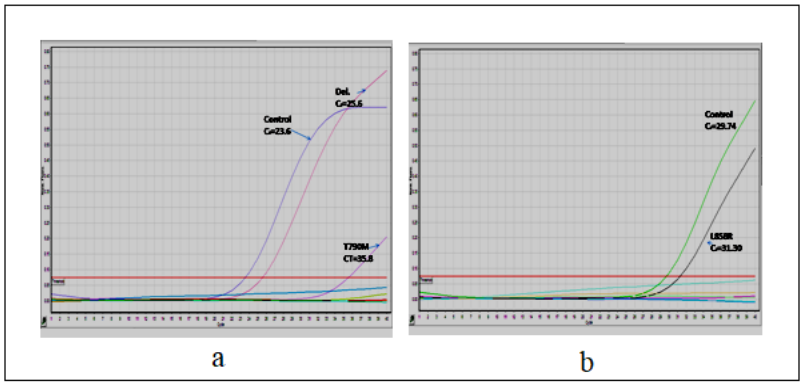

Figure 2. EGFR Amplification Curves. In Figure 2a, three curves were detected: control C_T = 23.6, T790M C_T = 35.58 (ΔC_T = 11.98 not within the cutoff range; a cutoff range of $\Delta C_T \leq 7.40$), and Del 19 C_T = 25.6 (ΔC_T = 2 within the cutoff range; a cutoff range of $\Delta C_T \leq 8.90$); therefore, this case was positive for deletion in exon 19. Meanwhile, two curves were detected in Figure 2b: control C_T = 29.74 and L858R C_T = 31.3 ΔC_T = 1.59 within the cutoff range (cutoff range of $\Delta C_T \leq 8.90$). Therefore this case was positive for exon 21.

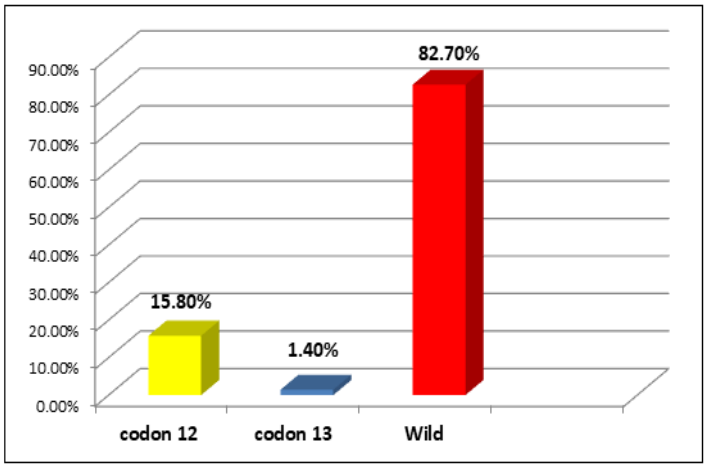

Figure 3. Frequency of K-RAS and Its Mutational Pattern

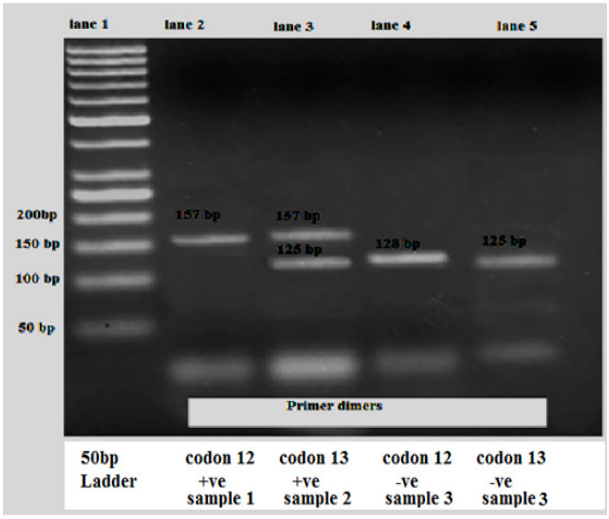

Figure 4. K-RAS Mutation Bands after RFLP Analysis. Lane 1: 50 bp ladder. Lane 2: 157 bp band, representing positive mutation for codon 12 (sample 1). Lane 3: 157 bp band, representing positive mutation for codon 13 allele and 125 bp band, representing WT for codon 13 allele (sample 2) (heterozygous sample). Lane 4: 128 bp band, representing WT for codon 12 (sample 3). Lane 5: 125 bp band, representing WT for codon 13 (sample 3).

ARMS is considered one of the most sensitive techniques as it could detect mutation as low as 0.1%–1% [45]. Using ARMS [46] and Scorpion [47] techniques, the frame deletion of exon 19 and point mutations in exon 21 in our study were the most common EGFR-detected mutations (12.2% and 8.2%, respectively), coinciding with most of the published data [28] [35] [44] [48,49]. In contrast, the G719X point mutation was only detected in two cases (1.4%), which is lower than that reported by Errihani (2013) [37] and Jazeih (2013 and 2015) [50,51].

For K-RAS mutations, codon 12 was the most common (15.8%) in our study, which agrees with previous findings [31,32] [52].

Detecting different mutational patterns of EGFR is crucial to consider during EGFR analysis, which contributes to variations in response to treatment, prognosis, and survival rates according to the different EGFR mutations. Better prognosis and long survival were associated with the mutation in exons 19 and 21, whereas poor prognosis was correlated with a mutation in exon 20 [53,54]. In contrast, the response rate to TKIs was improved in exons 19 and 21 mutations; exon 20 showed resistance to the therapy [38][55]. Also, exon 20 of the

Table 3. Association between EGFR, K-RAS Mutation Status, and Clinicopathological Feature of Patients

Clinicopathological feature		EGFR					K-RAS				
		Wild		Mutant		p value	Wild		Mutant		p value
		No	%	No	%	≤	No	%	No	%	≤
Age	≤59	52	75.4	17	24.6	NS	60	87	9	13	NS
(years)	>59	56	80	14	20	NS	55	78.6	15	21.4	NS
Gender	male	82	81.2	19	18.8	NS	83	82.2	18	17.8	NS
	female	26	68.4	12	31.6	NS	32	84.2	6	15.8	NS
Smoking	nonsmokers	37	71.2	15	28.8	NS	44	84.6	8	15.4	NS
	smoker	71	81.6	16	18.4	NS	71	81.6	16	18.4	NS
Performance status	1	77	78.6	21	21.4	NS	84	85.7	14	14.3	NS
	2	18	75	6	25	NS	18	75	6	25	NS
	3	6	85.7	1	14.3	NS	6	85.7	1	14.3	NS
Tumor	Low grade	64	88.9	8	11.1	NS	62	86.1	10	13.9	NS
Grade	high grade	19	45.2	23	54.8	<0.001	30	71.4	12	28.6	<0.05

EGFR gene and other downstream gene mutations showed resistance. K-RAS gene mutations are usually associated with TKI resistance [12] [15] [17].

In most studies, EGFR mutations were statistically significant with females and nonsmokers [19] [36] [56]. However, in our study, EGFR mutations were non-significantly more common in females and nonsmokers, which could be attributed to the fewer number of female patients compared with males (38 females vs. 101 males) and the fewer number of smokers compared with nonsmokers (52 nonsmokers vs. 87 smokers), coinciding with the studies of Unal (2013) [55] in Turkey and Ramadhan (2021) [38] in Iraq.

However, Dang (2020) [36] claimed that K-RAS mutation frequency was significantly higher in males than female patients and smokers than nonsmokers, which agreed with our study.

Conclusively, EGFR mutations were found in 22.3% of Egyptian patients with NSCLC/ADC and K-RAS in 17.3%, and the most frequent EGFR mutations detected in this study were in exons 19 and 21. Also, the prevalence of EGFR mutations was non-significantly higher in females than males and nonsmokers than smokers. Conversely, the K-RAS mutation was more common in males than females and smokers than nonsmokers. Furthermore, these frequencies were between the white and Asian populations, and they must be considered since they affect treatment and help predict the response of patients and their survival rates.

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