Epidermal growth factor receptor and *KRAS* mutations in lung adenocarcinoma: A retrospective study of the Lebanese population

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Received April 1, 2014; Accepted July 2, 2014

DOI: 10.3892/or.2014.3406

Abstract. Molecular genetic analysis of epidermal growth factor receptor (*EGFR)* and Kirsten rat sarcoma viral oncogene (*KRAS)* mutations in lung adenocarcinoma has become an integral part of lung cancer diagnosis and treatment; however, their prevalence varies with ethnicity. Little is know concerning their prevalence in Arab populations. In the present study, mutational analysis for *EGFR* and *KRAS* was performed on two cohorts of the Lebanese population. Lung adenocarcinoma cases (106) underwent mutational analysis for *KRAS* in exon 2, codon 12 and 13 and exon 3 codon 61 by reverse hybridization using the *KRAS* 12/13/61 StripAssay®. Subsequently, cases with no *KRAS* mutations underwent *EGFR* mutational analysis using the *EGFR* RGQ polymerase chain reaction (PCR) kits for real-time PCR on the Rotor-Gene Q 5-plex HRM. *KRAS* mutations were detected in 37.7% of 106 lung adenocarcinomas; 85% had a G>T substitution in codon 12 and 13 of exon 2, and 8.5% had *EGFR* mutations with exon 19 deletions (88.9%) and one case with L858R substitution in exon 21. *EGFR* mutations were significantly correlated with females, non-smokers and well differentiation of the tumor. This is the first study in an Arab population that reports the prevalence of both *EGFR* and *KRAS* gene mutations in lung adenocarcinoma using very sensitive mutational analysis techniques. Therefore, *EGFR* reflex testing should be implemented in the management of lung adenocarcinomas, while *KRAS* testing must await the identification of effective targeted therapy.

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Key words: lung adenocarcinoma, *EGFR*, *KRAS*, mutational analysis, reverse hybridization

Introduction

Lung cancer is the leading cause of cancer-related deaths in males and second after breast cancer in females. Its incidence is high and has a global age-standardized rate (ASR) of 47.4/100,000 and 18.6/100,000 for males and females, respectively (1). In the Arab countries, the incidence of lung cancer ranks after prostate, colon or bladder cancer in males and after breast and cervical cancer in females (2). The ASRs are 13.44/100,000 and 2.91/100,000 for males and females, respectively, but the highest incidence is reported in Tunisia followed by Bahrain. However, the largest number of cases occurs in North African countries, Egypt (20.6%), Morocco (20.1%), Algeria (15.4%) and Tunisia (10%), reflective of their high population. As for Lebanon, lung cancer incidence ranks third after Tunisia and Bahrain in males and second after Bahrain in females (3). The reported ASRs for lung adenocarcinoma in Lebanon are 27.3/100,000 and 10.2/100,000 for males and females, respectively (4).

For several decades lung cancer has been treated as a single disease, yet with greater knowledge of its molecular biology lung cancer is now considered a heterogeneous disease. Most important was the discovery of the association of epidermal growth factor receptor (*EGFR*) gene mutations and anaplastic lymphoma kinase (*ALK*) gene fusion with lung adenocarcinoma histology. These genetic aberrations have been linked to specific ethnicities and gender in never-smokers and have shown a good response to tyrosine kinase inhibitors (5-9). Consequently, mutational analysis of *EGFR* and *EML-ALK* has become a cornerstone in the management of patients with lung adenocarcinoma (10). Moreover, downstream of the *EGFR* pathway is the Kirsten rat sarcoma viral oncogene, or v-Ki-ras2 homolog (*KRAS*), known to occur in a wide range of cancers, with the highest prevalence in pancreatic (~90%),

colorectal (~60%) and primary lung adenocarcinomas (~60%), which can be mutated in lung cancer (11). When this mutation is detected, it is mutually exclusive of *EGFR* mutations but only rare and exceptional cases demonstrate both mutations (13), and a number of studies correlate its presence with resistance to anti-*EGFR* therapy and poor patient prognosis (11).

Lung cancer is notoriously correlated with smoking; therefore, the US and a number of European countries have implemented tobacco control programs and attained a decreased incidence worldwide (14,15). Unfortunately, in the Arab countries the incidence is increasing since smoking habits are increasing particularly among females and youth (3). Furthermore, in the hope of decreasing cancer-related mortality, almost all ethnic groups have been investigated for *EGFR* and *KRAS* gene mutation frequency in lung adenocarcinoma (16,17), but such a study has not been conducted in any Arab population including the Lebanese. Therefore, we aimed to evaluate the prevalence of *KRAS* and *EGFR* gene mutations in two Lebanese lung adenocarcinoma cohort groups and to correlate the findings with the clinical and pathologic features, including age, gender, smoking history and histological grade.

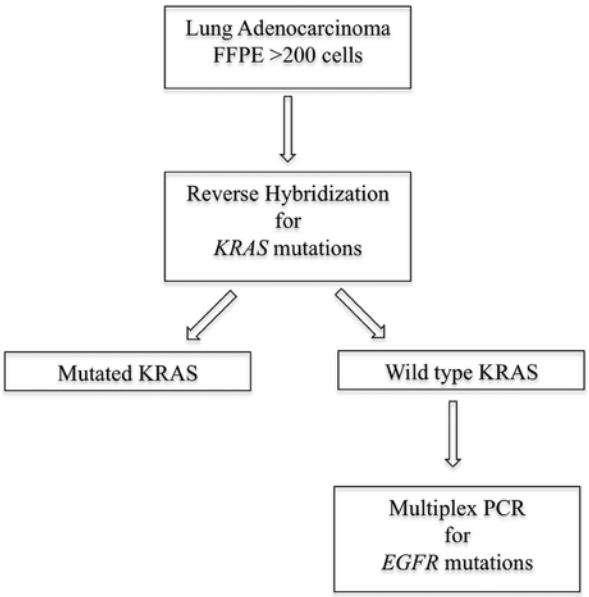

Figure 1. Algorithm for lung adenocarcinoma molecular analysis. FFPE, formalin-fixed paraffin-embedded.

Materials and methods

The Institutional Review Boards of the American University of Beirut Medical Center and Hammoud Hospital University Medical Center approved this study. Both boards waived the need for written patient informed consents.

Patient selection. Patient cases (242) diagnosed clinically with primary lung adenocarcinoma (AC) and 150 with non-small cell carcinoma-not otherwise specified (NSCLC-NOS) were retrieved from the archives of the Pathology Departments at the American University of Beirut Medical Center and Hammoud Hospital University Medical Center in Lebanon from the year 2001 to 2010. Of the 150 NSCLC-NOS cases, 91 had an adequate tumor size (>1 mm) for further subtyping by immunohistochemical staining and 37 cases were diagnosed as 'favoring primary lung AC'. Moreover, to ensure good quality and quantity of extracted DNA for mutational analysis, all 279 primary lung AC cases were then evaluated microscopically for tumor size. The cases with >200 tumor cells were included; thus, 106 primary lung AC cases were selected for *EGFR* and *KRAS* mutational analysis. The clinicopathologic information pertaining to patient age at diagnosis, gender, history of smoking, grade and TNM stage was retrieved for all cases, and none of the patients in this study received chemotherapy prior to specimen resection (lobectomy) or biopsy.

Immunohistochemistry. Three histological sections of 3-μm thickness were prepared from formalin-fixed paraffin-embedded (FFPE) tissue blocks of the NSCLC-NOS cases. Immunomarker detection was performed using antibodies against TTF-1 (thyroid transcription factor-1) (1:200 dilution; Novocastra; SPT24), napsin A (1:400 dilution; Novocastra; IP64) and p63 (Ready-to-use; Novocastra; 7JUL). Positive staining for TTF-1 and/or napsin A was used for identifying AC and p63 for squamous cell carcinoma. The immunomarkers were considered positive if at least 10% of the tumor cells stained. Cases with <10% staining and no focal areas of positive staining were interpreted as negative. Appropriate positive and negative controls were included. Immunostaining by TTF-1 of entrapped normal lung epithelium or napsin A of pulmonary macrophages was ignored. Immunohistochemistry was performed using the Leica Bond-Max autostainer (Leica Microsystems Inc., Buffalo Grove, IL, USA) with the manufacturer's preset timed reagents.

Mutational analysis for KRAS and EGFR. FFPE tissue blocks of the included 106 lung adenocarcinoma cases were collected for *KRAS* and *EGFR* mutational analysis according to the algorithm (Fig. 1).

DNA extraction from FFPE. Eight 10-μm FFPE tissue ribbons were obtained from each block, and lysis was allowed overnight at 65˚C with 1.5 μl proteinase K (20 mg/ml) and 400 μl cell lysis solution (Qiagen; 158906). DNA was extracted following a standard protocol and quantified using the NanoDrop ND-1000 spectrophotometer. The resultant DNA was stored at 4˚C until utilized for reverse hybridization and multiplex real-time polymerase chain reaction (PCR) procedures.

Reverse hybridization for detection of KRAS mutations. Detection of *KRAS* mutations in exon 2, codon 12 and 13 and exon 3 codon 61 was performed by reverse hybridization using the *KRAS* 12/13/61 StripAssay® (ViennaLab Diagnostics GmbH, Vienna, Austria). This technique involves three steps: PCR amplification using biotinylated oligonucleotide primers, hybridization of the amplified products to a strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines and placed in a shaking water bath at 45˚C and identification of bound biotinylated sequences using streptavidin-alkaline phosohatase and color substrates performed at room temperature. Bands were then analyzed visually. Cases that did not exhibit these mutations were then submitted for

Table I. Overall clinical characteristics and association with *KRAS* and *EGFR* mutations.

Variables	Overall n (%)	*KRAS* mutation n (%)	No *KRAS* mutation n (%)	P-value	*EGFR* mutation n (%)	No *EGFR* mutation n (%)	P-value
Age (years)				0.172			0.232
Mean ± SD	62.1±10.4	64.0±8.7	61.0±11.2		57.8±8.8	62.3±10.4	
Gender				0.942			0.005[a]
Female	34 (32.1)	13 (32.5)	21 (31.8)		7 (77.8)	27 (28.4)	
Male	72 (67.9)	27 (67.5)	45 (68.2)		2 (22.2)	70 (71.6)	
Tumor differentiation				0.207			<0.001[a]
Poor	66 (62.3)	25 (62.5)	41 (62.1)		5 (55.6)	61 (62.9)	
Moderate	35 (33.0)	15 (37.5)	20 (30.3)		0 (0.0)	35 (36.1)	
Well	5 (4.7)	0 (0.0)	5 (7.6)		4 (44.4)	1 (1.0)	
Smoking				0.286			0.003[a]
Yes	59 (55.7)	23 (57.5)	36 (54.6)		1 (11.1)	58 (59.8)	
No	18 (17.0)	4 (10.0)	14 (21.2)		5 (55.6)	13 (13.4)	
Not available	29 (27.4)	13 (32.5)	16 (24.2)		3 (33.3)	26 (26.8)	
Tumor size (T) (cm)				0.389			0.881
≤3	12 (11.3)	6 (15.0)	6 (9.1)		1 (11.1)	11 (11.3)	
>3	31 (29.3)	9 (22.5)	22 (33.3)		2 (22.2)	29 (29.9)	
Not available	63 (59.4)	25 (62.5)	38 (57.6)		6 (66.7)	57 (58.8)	
Lymph node (N)				0.879			0.424
Yes	19 (17.9)	7 (17.5)	12 (18.2)		0 (0.0)	19 (19.6)	
No	21 (19.8)	7 (17.5)	14 (21.2)		2 (22.2)	19 (19.6)	
Not available	66 (62.3)	26 (65.0)	40 (60.6)		7 (77.8)	59 (60.8)	
Metastasis (M)				0.658			0.792
Yes	20 (18.9)	6 (15.0)	14 (21.2)		2 (22.2)	18 (18.6)	
No	23 (21.7)	10 (25.0)	13 (19.7)		1 (11.1)	22 (22.7)	
Not available	63 (59.4)	24 (60.0)	39 (59.1)		6 (66.7)	57 (58.8)	

[a]Significant difference. *KRAS*, Kirsten rat sarcoma viral oncogene; *EGFR*, epidermal growth factor receptor.

EGFR exons 18-21 mutational analysis, knowing that *KRAS* and *EGFR* mutations are predominantly mutually exclusive.

Multiplex real-time PCR for detection of EGFR mutations. *EGFR* mutational analysis was performed using the *EGFR* RGQ PCR kits (Qiagen, Valencia, CA, USA), that enables detection of 29 somatic mutations in the *EGFR* gene including: 19 deletions in exon 19, T790M-exon 20, L858R-exon 21, L861Q-exon 21, G719X-exon 18, S768I-exon 20 and 3 insertions in exon 20. The kit utilizes two technologies, amplification refractory mutation system (ARMS) technology and Scorpions dual primer probes available for detection of *EGFR* mutations using real-time PCR on the Rotor-Gene Q 5-plex HRM instrument (Qiagen). This analysis is performed in two steps: the first step is to perform a control assay in order to assess the total DNA in the sample and the second step is to complete the assay for the presence or absence of mutated DNA. This procedure was completed as per the manufacturer's instructions manual. We considered this technology because of its high sensitivity particularly for samples with low levels of tumor DNA, such as in small lung biopsy samples.

Statistical analysis. Age, gender, smoking history, grade and TNM stage were summarized using means and standard deviation for age and frequency distribution for the other 4 variables. Those variables were compared between patients with *KRAS* mutation and those without *KRAS* mutation using the independent t-test for age (or Wilcoxon rank sum test for small sample size) and the Chi-squared test or Fisher's exact test (when cell counts were small) for the other 4 variables. Similar comparisons were made between patients with an *EGFR* mutation and those without mutations.

Results

The overall clinical and pathological data of the 106 primary lung adenocarcinoma cases demonstrated a mean age of 62 years and a male to female ratio of 2:1. The majority of patients were smokers (55.7%), although we could not determine the smoking history data for 27.7%. A total of 56.2% of the tumors were poorly differentiated when applying the three-tier grading system. TNM staging data were available for 45% of the cases, which limited our statistical analysis, but more

Table II. Summary of *KRAS* mutations in exon 2.

KRAS mutation	No. of cases
c.34G>T, p.G12C	19
c.34G>A, p.G12C	1
c.35G>C, p.G12A	11
c.35G>A, p.G12A	2
c.35G>A, p.G12D	5
c.35G>T, p.G12V	2
c.37G>T, p.G13C	2
c.38G>A, p.G13A	2
c.38G>A, p.G13D	2

KRAS, Kirsten rat sarcoma viral oncogene.

Table III. Summary of *EGFR* mutations.

EGFR mutation	No. of cases
Exon 18	0
Exon 19 deletions	8
Exon 20	0
L858R-exon 21	1

EGFR, epidermal growth factor receptor.

tumors had a size larger than 3 cm, while approximately half of the cases had lymph node and/or distant metastasis (Table I).

The prevalence of *KRAS* mutations was detected in 37.7% of the primary lung AC cases. The majority (85%) had a G>T substitutions in codon 12 of exon 2 and 6 cases had this substitution in codon 13 (Table II). An A>G substitution in codon 61 of exon 3 was detected in one case. All of the *KRAS* mutations were single except for 5 cases that exhibited double mutations. The *KRAS* mutations were predominant in males (67.5 vs. 32.5% in females) and in smokers (57.5 vs. 10.0% in non-smokers); however, no statistical significance could be concluded (p=0.942 and p=0.286, respectively). The majority of the *KRAS*-mutated tumors were poorly differentiated (62.5%) and had a tumor size larger than 3 cm, but the frequency of lymph node or distant metastasis was not significantly higher when compared to the lung adenocarcinoma cases with no *KRAS* mutation (Table I).

EGFR mutations were detected in 9 (8.5%) of the lung adenocarcinoma patients with no *KRAS* mutations. The most common mutations (8 cases, 88.9%) were deletion in exon 19 while one case had a substitution L858R in exon 21 (Table III). Predominance of *EGFR* mutations was significant in females (p=0.005), non-smokers (p=0.003) and well differentiated tumors (p<0.001). TNM data were not available for all the 9 cases; therefore, we could not draw a significant correlation (Table I).

In summary, we identified mutations in 46.2% of the lung adenocarcinoma cases distributed into 37.7% with *KRAS* mutations and 8.5% with *EGFR* mutations (Table IV).

Table IV. Summary of the *EGFR* and *KRAS* mutations in primary adenocarcinoma.

	Mutations		
	EGFR n (%)	*KRAS* n (%)	Total n (%)
Cases	9 (8.5)	40 (37.7)	49 (45.2)

KRAS, Kirsten rat sarcoma viral oncogene; *EGFR*, epidermal growth factor receptor.

Discussion

Molecular genetic analysis of lung adenocarcinoma has become an integral part of lung cancer diagnosis and present treatment (18,19). The most commonly detected driver mutations are those involving *KRAS*, *EGFR* and *EML4-ALK* genes. Moreover, *EGFR* tyrosine kinase inhibitors (TKIs), gefitinib, erlotinib and afatinib, are considered as first-line targeted therapy for advanced (locally or metastatic) lung adenocarcinoma. However, the prevalence of these aberrations varies with gender, ethnicity and smoking history (20). Consequently, lung adenocarcinomas have been extensively profiled for genetic aberrations in most ethnic groups. In the Arab population, only one study evaluated *EGFR* genetic aberrations in 34 Saudi lung cancer patients (21). In the present study, 106 cases of lung adenocarcinomas from Lebanon were analyzed for mutations in *KRAS* and *EGFR*.

EGFR mutations were detected in 8.5% of the lung adenocarcinomas, a frequency higher than that in the Saudi population, comparable to that of some European countries, but much lower than that of Asian countries. The highest reported frequency of *EGFR* mutations is in Asian, non-smoker females (Taiwan, 57.3%) (22). However, the Europeans reported much lower frequencies and Szumera-Ciećkiewicz *et al* reviewed the results from 19 European studies and showed a range of 2.6% (in Italy) to 39% (in Germany) (23). In the US, a study of 3026 cases by Dogan *et al* demonstrated a frequency of 20% (24) and Reinersman *et al* reported a frequency of 19% in African-Americans (25). In Latin America, the average frequency is 33.2% with the lowest in Argentina (19.3%) and the highest in Peru (67%). This reported frequency is correlated with the high rate of Asian migration (26). To the best of our knowledge, in the Middle East and the Levant countries, only one study from Saudi Arabia has been published. This study applied direct DNA sequencing to 34 lung cancer cases and detected the *EGFR* mutation in one lung adenocarcinoma (3%) (21).

The analysis of *EGFR* mutation subtypes demonstrated a predominance of deletions in exon 19 and only one of the 9 cases had an L858R substitution in exon 21. These findings are comparable to the reported frequency of *EGFR* mutation subtypes: in-frame deletions in exon 19 (44% of all mutations), missense point mutations (L858R substitution) in exon 21 (41% of all mutations), in-frame duplications/insertions in exon 20 (5% of all mutations) and G719X (X indicates A, C or S) substitution in exon 18 (4% of all mutations) (27). It is impor-

Table V. Frequency of *KRAS* and *EGFR* mutations reported from different countries and ethnicities.

Country	*KRAS* (%)	*EGFR* (%)	Smoking history	N[a]	Year (ref.)
Australia	17.0			108	1998 (38)
Saudi Arabia		2.94		34	2006 (21)
Hong Kong	9.8	54.0	Correlated	215	2006 (39)
Korea	7.3	17.4		55	2007 (40)
USA	21.2		Not correlated	482	2008 (31)
Taiwan	5.03	57.3	Not correlated	159	2008 (22)
Japan	12.6	49.4		254	2009 (41)
Korea	9.6	24.0		94	2009 (42)
Italy	17.9	12.6		411	2009 (43)
USA	23.0	10.0	Correlated	345	2010 (36)
Argentina		19.3	Correlated	244	2011 (26)
Columbia	17.1	24.8	Correlated	322	2011 (26)
Mexico	16.0	31.2	Correlated	381	2011 (26)
Peru	16.8	67.0	Correlated	381	2011 (26)
China	8.0	41.0	Correlated	861	2012 (44)
USA	26.0	20.0	Correlated	2529/3026[b]	2012 (24)
Japan	16.5	41.7	Correlated	182	2012 (45)
Netherlands	36.9	10.6	Correlated	662	2012 (46)
Brazilian	14.6	30.4	Correlated	207	2012 (47)
China	5.9			1935	2013 (48)
Czech Republic	21.0			233	2013 (49)
Western Turkey		42.6	Correlated	48	2013 (50)
Europe		2.6-39[c]	Correlated	23-147	2013 (23)
Korea		39.0	Correlated	502	2013 (51)
Lebanon	37.7	8.5	Not correlated	106	Present study

[a]N, no. of cases evaluated for *KRAS* and/or *EGFR*; [b]2529: for *KRAS*, 3026: for *EGFR*; [c]Lowest in Italy, highest in Germany. *KRAS*, Kirsten rat sarcoma viral oncogene; *EGFR*, epidermal growth factor receptor.

tant to subtype these mutations since they respond differently to *EGFR* TKIs (28). For example, the T790M mutation in exon 20 correlates with drug resistance and relapse (29). In addition, we demonstrated a significant correlation between *EGFR* mutations and females, non-smokers and well differentiation of the tumor. These findings are in concordance with the reported prevalence of these mutations in females more than in males and in never-smokers than in smokers and to their correlation with a better prognosis (30).

KRAS mutations were detected in 37.7% of the lung adenocarcinomas. Similar to *EGFR* mutations, the prevalence of *KRAS* mutations in lung adenocarcinoma varies with ethnic groups as well, but is generally mutually exclusive with *EGFR* mutations (13,31). An overview of *KRAS* and *EGFR* mutation frequency from different countries presented in Table V demonstrates an inverse *EGFR* mutation frequency as compared to that of *KRAS*. The lowest values were reported in Asians (Taiwan: *KRAS,* 5.03%; *EGFR,* 57.3%), while the highest *KRAS* prevalence was reported in Europeans (The Netherlands: *KRAS,* 36.9%; *EGFR,* 10.6%).

In addition, we found that *KRAS* mutations were predominantly in exons 2 and 3 with 85% involving codon 12, thus concordant with that reported by Bos, where 97% of *KRAS* mutation subtypes are substitutions of *G→T* and *G→C* in codon 12 of exon 2 in addition to the missense point mutations in codons 61 that prevent GTP cleaving, thus transforming the gene into an oncogene (32). However, we could not demonstrate a correlation between smoking and the type of substitution and the subtype of the mutation. Such a correlation was described by Riely *et al* where they demonstrated that the *G→T* and *G→C* transversions are present in both smokers and non-smokers, while a *G→A* transversion is more common in non-smokers (12). Moreover, we detected more *KRAS* mutations in males, smokers and poorly differentiated tumors, but this observation did not demonstrate a statistically significant correlation. Several other studies have shown that *KRAS* mutations are higher in males and smokers as compared to females and never-smokers (33), predict a worse prognosis (34,35), and show a low response to adjuvant chemotherapy and *EGFR*-TKI treatment in metastatic lung adenocarcinoma (7). Unfortunately, because it leads to loss of enzymatic activity, effective targeted therapy is more difficult to achieve as compared to a gain of functional mutation. Currently, clinical trials targeting downstream effector molecules are being conducted, but no effective targeted therapy against *KRAS* mutations has been achieved thus far.

Two mutational analysis techniques were used in this study. The Scorpion amplified refractory mutation system (ARMS) multiplex real-time PCR was used for the detection of *EGFR* mutations. Although this technique does not detect mutations comprehensively, it covers all the common 29 *EGFR* mutations in exons 18-21. It is highly sensitive (1%) as compared to direct Sanger sequencing (25%) of mutant DNA (36). For *KRAS* mutations we used reverse hybridization (StripAssay, ViennaLab), which is considered most analytically sensitive when compared to direct sequencing, pyrosequencing, high resolution melting analysis and the TheraScreen DxS kit (37). This technique can detect 13 mutations in the *KRAS* codon 12, 13 and 61. The high sensitivity of both techniques was necessary to overcome the tissue limitation of small lung biopsy material that constituted the majority of our cases. However, some biopsy material was too scant to be included in this study thus constituting a limitation for this study. In addition, the unavailability of adequate smoking history data for all cases, and being retrospective in nature are additional limitations.

In summary, this is the first study in an Arab population to report the prevalence of both *EGFR* and *KRAS* gene mutations in lung adenocarcinoma using sensitive mutational analysis techniques. We showed that the frequency of *EGFR* in lung adenocarcinoma was 8.5% and that it was more common in women, non-smokers and well-differentiated tumors. In addition, we found that *KRAS* was highly prevalent in the Lebanese population, but we could not significantly correlate it with smoking; however, it remains a possible major cause. Therefore, we conclude that *EGFR* reflex testing should be implemented as per the recommendation of the College of American Pathologists, International Association for the Study of Lung Cancer and Association for Molecular Pathology (10). While *KRAS* testing is useful for understanding the molecular biology of adenocarcinoma, the clinical utilization of this information must await the identification of effective targeted therapy.

Acknowledgements

The authors are grateful for the generous funding support provided by the Lebanese National Council for Scientific Research (LNCSR), grant no. PALM. GZ.09.

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