Journal of Clinical and Biomedical Sciences

Journal homepage: www.jcbsonline.ac.in

Original Article

Detection of *EGFR* Mutations in Bronchial Wash from Iraqi patients with non-small Cell Lung Cancer (NSCLC)

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Received: 28th July -2014 Accepted: 16th August-2014 Published: 30th September - 2014

Abstract

Background: Non-small cell lung cancer (NSCLC) is caused of 85% of all lung cancers. Among the most important factors for lung tumor growth and proliferation are the tyrosine kinase receptors that coded by the epidermal growth factor recep-tor (*EGFR*) gene. Activation of *EGFR* ultimately leads to developing of lung cancer. The present study was undertaken with an objective to detect *EGFR* mutations in bronchial wash from Iraqi patients with NSCLC before treatment. ***Methods:*** DNA was extracted from bronchial wash samples collected from 50 patients with NSCLC by using a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany). Then, *EGFR* mutations were determined by using real-time RCR combined with two technologies**,** Amplification Refractory Mutation System (ARMS) and Scorpions. ***Results:*** A point mutation, G719X, in exon−18 with three different profiles, G719A, G719S, and G719C was significantly diffused in *EGFR*. L858R in the same exon and T790M in exon−20 was also detected. While no deletions in exon −19, and no substitutions or insertions in exon −20 were found. Moreover, no significant differences (*P≤0.05*) in *EGFR* mutations were seen between males (28.57%) and females (30.76%). In contrast, *EGFR* mutations were significantly (*P≤0.05*) prevalent in smoker's males (26.6%) than females 6.6%). ***Conclusion:*** Using the bronchial wash samples was efficient for detection of mutations in lung cancer. Moreover, Iraqi patients with NSCLC were discriminated in *EGFR* genotype; the point mutation G179X in exon−20 was dominant and L858R in the same exon and T790M in exon−20 were detected while no mutations in exon− 19 and −20 were investigated.

Key-words *EGFR* mutations, Non- small cell lung cancer, bronchial wash, exon

Introduction

Lung cancer is the uncontrolled growth of abnormal cells, it can start in the cells lining of bronchi and parts of the lung such as the bronchioles or alveoli. They start as areas of pre-cancerous changes in the lung [(1)]. This disease continues to be the most common cancer worldwide in terms of both incidence and mortality. In Iraq, there were 1570 cases of lung cancer accounting for 10.29% of all newly diagnosed cancer cases; it was affected 1151 males and 419 females [(2)]. Lung cancer comprised of two major clinic-pathological cat-egories, small-cell lung cancer (SCLC) and non-small-cell lung carcinoma (NSCLC) [(3)]. Non-small cell lung cancer (NSCLC) is the major cause of cancer deaths [(4)] it caused about 85% of all lung cancers. NSCLC divided into three major histological subtypes: adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma [(5)].

patients with NSCLC that may provide the opportunity for treatment.Previous studies reported several environmental, hormonal, and viral risk factors that causing NSCLC particularly in non-smokers [(6-8)]. Moreover, alternations in tumor suppressor genes and/or proto-oncogenes are contributed to develop this disease. Notable, activation of epidermal growth factor receptor (*EGFR*), an a proto-oncogene represents one of four tyrosine kinase receptors [(9)], has been frequently identified in the development and progression NSCLC [(10, 11)]. In NSCLC adenocarcinomas, somatic mutations in *EGFR* were identified in approximately 10% of tumors from patients in the United States and in 30-50% of tumors from patients in Asia [(12)].

In Iraq, the patients who had NSCLC were treated with *EGFR*-tyrosine kinase inhibitors such as gefitinib or erlotinib but unfortunately it showed low response.

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Quick access Code

Thus, this study was aimed to detect the specific somatic mutations in the several exons of *EGFR* and determine the genotyping of *EGFR* in Iraqi patients with NSCLC that may provide the opportunity for treatment.

Materials and Methods

A cohort of 50 patients (25 males and 25 females) diagnosed with non small cell lung cancer (NSCLC) before treatment were attended to different Hospitals in Baghdad such as National center for early detection of tumors, Medical city, and Ghazi al-Hariri, from November 2012 to July 2013. The median age of patients was 65 years for males and 50 years for females. Among those patients, 22 had adenocarcinoma, 14 had squamous cell carcinoma, and 14 had large cell carcinoma. Regarding smoking history, 24 of patients were non-smoker, 16 were former, and 10 were currently smoker. This study was conducted with the approval of the appropriate ethical review boards based on the recommendations of Declaration of the Ministry of Health involving human subjects.

Collection of samples: Bronchial wash samples from 50 patients with NSCLC were collected. In addition, buccal swabs from 10 of healthy individuals were collected and used as a control.After bronchoscopy was done for patients with NSCLC, 12 to 15 ml of bronchial wash were collected and immediately centrifuged at 675 ×g for 5min., the pellets were resuspended in 200µl of 95% ethanol as a fixative solution**.** Then**,** the DNA was extracted and purified using a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The resulting DNA was eluted in 50 µL of sterile bidistilled buffer. The concentration and purity of the extracted DNA were determined by nanodrop. The extracted DNA was stocked at −20°C until use.

On the other hand, to each swab collected from 10 healthy individuals, 300µl of lyses buffer were added, mixed in thermo mixer at 70ºC for 40min., centrifuged at 1000rpm for 2sec, and then the DNA was extracted and purified by using Prep Filter ^{TM} kit (Applied Biosystem, UK) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were determined by nanodrop. The extracted DNA was stocked at −20°C until use.EGFR Scorpion Kit (DxS Ltd., Manchester, United Kingdom) was used which combined two technologies**,** Amplification Refractory Mutation System (ARMS) and Scorpions on the Rotor-Gene Q**,** to detect mutations in real-time PCR reactions. Eight kinds of scorpion primers for detection of G719X, del 19, S768I, Ins, T790M, L858R,L861Q, and wild type in exon 18,19, 20, and 21 were designed and synthesized by DxS. All reactions were done in 25µl using 1 µl of template DNA, 7.5 µl of reaction buffer mix, 0.6 µL of primer mix, and 0.1 µl of Taq polymerase. All reagents are included in this kit. Real-time PCR was carried out using SmartCycler II (Cepheid, Sunnyvale, CA) under the following conditions: initial denaturation at 95°C for 15 minutes, 40 cycles of 95°C for 30 seconds, and 60°C for 60 seconds with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was done with Cepheid SmartCycler software (Ver. 1.2b). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represented the point of maximum curvature of the growth curve. Both Ct and maximum fluorescence (Fl) were used for interpretation of the results. Positive results were defined as follows: Ct ≤45 and Fl ≥50. These analyses were done in duplicate for each sample.

Statistical analysis

Two samples, the Statistical Analysis System-SAS (2010) and Chi-square test were used to analyze the data.

Results

The concentration of DNA that extracted from bronchial wash samples of 50 patients with NSCLC was measurement; it was at a median 92ng/ml (range 4–180ng/ml) with purity ranged from 1.7 to1.9. While the median concentration of DNA that extracted from buccal swabs collected from 10 healthy individuals (as control) was 55ng/ml (range 29–190ng/ml) with purity ranged from 1.72 to 1.87.

Preliminary experiment to evaluate the quality of extracted DNA from bronchial wash and buccal swab samples was done by using Scorpion-ARMS techniques. The results showed 3 of 10 (30%) buccal swab samples and 23 of 50 (48%) bronchial wash samples were failed. Thus, the extracted DNA from 27 samples of bronchial wash and 7 samples from buccal swabs that given a good quality were used in this study.Thereafter, the type, number, and percentage of *EGFR* mutations in 27 patients with NSCLC were determined (Table 1 and Table 2).

Exon	Type of mutations	Mutations	No. of mutations	Percentage (%)
18	Point mutation	G719X	5	35.7%
19	In-frame deletion	deletions	0	0
20	substitution	S768I	0	0
	insertion	insertions	0	0
	Secondary point mutation	T790M	1	5.5%
21	point mutation	L858R	2	8%
		L861Q	0	0

Table1: Type of *EGFR* gene mutation in Iraqi patients with *NSCLC* with different Exons.

Exon	Mutations	Change in base pair
18	G719X G719A G719S G719C	2156G>C 2155G>A 2155G>T
19	deletions	2235_2249del15 2235_2252>AAT (complex) 2236_2253del18 2237_2251del15 2237_2254del18 2237_2255>T (complex) 2236_2250del15 2238_2255del18 2238_2248>GC (complex) 2238_2252>GCA (complex) 2239_2247del9 2239_2253del15 2239_2256del18 2239_2248TTAAGAGAAG>C (complex) 2239_2258>CA (complex) 2240_2251del12 2240_2257del18 2240_2254del15 2239_2251>C (complex)
20	S768I	2303G>T
	insertions	2307_2308ins9 2319_2320insCAC 2310_2311insGGT
	T790M	2369C>T
21	L858R	2573T>G
	L861Q	2582T>A

Table2: Alteration of bases in EGFR gene in Iraqi patients with NSCLC

The results indicated a significant (*P≤0.05*) percentage (35.7%) of point mutation of G719X in exon−18, and it appeared in three different profiles G719A, G719S, and G719C. Another point mutation was detected in exon−21, it was L858R which formed 8%. Remarkably, this exon was seemed free of L861Q mutations. Whereas, the secondary point mutation, T790M, in exon−20 was found at low percentage (5.5%). Notable, *EGFR* gene in Iraqi patients had no deletions in exon −19 as well as no substitution or insertion mutant (S768I) was reported in exon −20. In addition, the results in Fig. 1 indicated no mutations in *EGFR* gene were detected in the DNA of healthy individuals that used as a negative control.

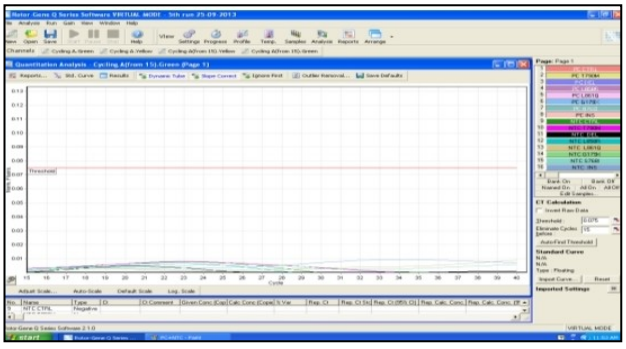

Figure 1: No mutation in EGFR gene in normal individuals

On the other hand, determination of *EGFR* mutations according to the Iraqi gender with NSCLC was investigated. This study involved 14 males and 13 females, the results in Table 3 indicated that 4 of 14 (28.57%) males had *EGFR* mutation represent one point mutation G179X in exon-18. While 30.76% of females had *EGFR* mutations; it represent G719X, T790M in exon −20, and L858R in exon −21; the number of these mutations was 1, 1, and 2 respectively.

While several mutations besides G719X in exon −18 were reported in females with NSCLC, it was T790 Min exon −20 and L858R in exon −21, the number of these mutations were 1, 1, and 2, respectively.Moreover, *EGFR* gene mutations in 15 smokers with NSCLC were investigated. The point mutation G719X in exon −18 was reported in 4(26.6%) males and one (6.6%) female as shown in Table 3.

Gender	Total No.	Positive		Negative		Exon	Mutation	No. of Mutations
		No.	%	No.	%			
Male	14	4	*28.57	10	71.43	18	G719X	4
Female	13	4	30.76	9	69.23	18	G719X	1
						20	T790M	1
						21	L861Q	2
Chi square=0.016, D.F. 1; P ≤0.05 *No significant differences								

Table3: mutation in *EGFR* gene according to the Iraqi gender with *NSCLC*

Discussion

Our finding showed that extracted DNA from 3 samples of buccal swab and 23 samples of bronchial wash were failed when assessed by using Scorpion-ARMS techniques. This may return to the inhibitors or to the chemical processes involved in the fixation and storage process which may cause DNA damage and then result in failed reactions [13].This damage may reduce the amplification strength of DNA polymerase enzymes as often encounter 'blocks' the DNA template. Moreover, the miss incorporation of nucleotides is common with a degraded DNA template, which further reduces the efficiency of amplification [14]. Although the most common *EGFR* sensitizing mutations in patients with NSCLC from Japan and Turkey are found in exon-19 as microdeletions, and in exon-21as point mutation of L858R, these two types of mutations comprise up to approximately 85 to 90% of known *EGFR* activating mutations in NSCLC, while G719X mutation comprise 5% , and T790M mutation comprise 5% [15-18]. However, in this study a significant (*p≤0.05*) dominant of point mutantion G719X in exon −18 was recorded, it acheived 35.5%. The next was L858R in exon −21, it was formed 8%, and then secondary point mutation T790M in exon −20 was found at low percentage (5.5%). From these results it can be suggested that Iraqi patients had a specific genotyping with point mutations found frequently in exons−18, 21, and 20. Whereas no deletions, insertions, or substitutions were determined in *EGFR* gene. In addition, this study involved the investigation of *EGFR* mutations in Iraqi gender with NSCLC. The results showed no significant differences (*p≤0.05*) in *EGFR* mutations between males (28.57%) and females (30.76%). Notable, smoking-related lung cancer is the leading cause of cancer deaths in both men and women in united state. Thus this study investigates the relationship between smoking and NSCLC in Iraqi patients. The results showed that the point mutation G719X in exon −18 was frequently found in smokers with significantly (*P≤0.05*) prevalence in males than females.

However, the molecular mechanisms underlying the induction of lung cancer by cigarette smoke are still poorly understood. EGFR activation induced by cigarette smoke was in fact independent of ligand binding. Specifically, one of the major reactive oxidants in the gas phase of cigarette smoke, hydrogen peroxide (H_2O_2), caused aberrant phosphorylation and activation of the EGFR [19] in human airway epithelial cells. The abnormal phosphorylation of the receptor in cells exposed to H_2O_2- induced oxidative stress also acquired an aberrant activated conformation that impaired canonical dimerization of EGFR [16]. This activated EGFR was neither ubiquitinated nor subsequently degraded due to its inability to bind the E3-lygase, c-Cbl. This allowed EGFR to remain active for a longer period at the plasma membrane, thereby causing prolonged survival signals that contributed to uncontrolled cell growth [20].

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