ABSTRACT

Context: Nonsmall cell lung carcinoma (NSCLC) is the most frequently diagnosed form of lung cancer in Kuwait. NSCLC samples from Kuwait have never been screened for epidermal growth factor receptor (EGFR) gene aberration, which is known to affect treatment options.

Aims: This study investigated the feasibility of using fine-needle aspiration (FNA) material for mutational screening, and whether common EGFR mutations are present in NSCLC samples from Kuwait.

Settings and Design: Eighteen NSCLC samples from five Kuwaitis and 13 non-Kuwaitis were included in this study.

Materials and Methods: DNA was extracted from FNA cell blocks and screened for EGFR gene mutations using peptide nucleic acid (PNA)-clamp assay, and EGFR gene amplification using fluorescent in situ hybridization (EGFR-FISH). EGFR protein expression was assessed using immunohistochemistry.

Results: Five EGFR mutations were detected in five non-Kuwaiti NSCLC patients (27.8%). EGFR gene amplification was evident in 10 samples (55.5%) by direct amplification or under the influence of chromosomal polysomy. Four samples had EGFR mutations and EGFR gene amplification, out of which only one sample had coexisting EGFR overexpression.

Conclusions: Given the evidence of EGFR gene alterations occurring in NSCLC patients in Kuwait, there is a need to incorporate EGFR gene mutational screen for NSCLC patients to implement its consequent use in patient treatment.

Key words: Epidermal growth factor receptor (EGFR); fine-needle aspiration (FNA); lung adenocarcinoma; mutational screen; nonsmall cell lung carcinoma (NSCLC)

Introduction

Lung cancer is the leading cause of worldwide cancer mortality with a gradual increase in its incidence worldwide.[1] In Kuwait, lung cancer constitutes 13% of all cancer cases and is the fourth most common cancer. Kuwait has an incidence rate of 17 for males and 8.4 for females per 100,000 individuals.[2] Among the frequent lung cancer forms diagnosed in Kuwait, one is nonsmall cell lung carcinomas (NSCLC). NSCLC groups three major lung cancer subtypes; adenocarcinoma (AD), large cell carcinoma (LCC), and squamous cell lung carcinoma (SqCLC), which are discerned by the positive expression of specific markers including AD-specific thyroid transcription factor 1 (TTF-1) and p63 expression in SqCLC.[3] There are several identified molecular markers that guide in targeted therapeutics and predict the outcome in NSCLC.[4] The major molecular markers identified that have a high prevalence in

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NSCLC include echinoderm microtubule-associated protein-like 4 (EML4), anaplastic lymphoma kinase (ALK), epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog (KRAS), and v-raf murine sarcoma viral oncogene homologue B1 (BRAF). The identification of EGFR mutation-positive NSCLC permits the use of tyrosine kinase inhibitors (TKI) as an effective treatment. EGFR (gene ID:1956) is a transmembrane glycoprotein receptor with an extracellular binding domain and a cytoplasmic tyrosine kinase domain. Growth factor binding induces receptor dimerization and tyrosine autophosphorylation, resulting in a cellular relay of cell proliferation signals. Cancer cells with EGFR mutations not only possess a growth advantage over those with wild type EGFR but also show increased sensitivity to anti-EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib. EGFR mutations associating with NSCLC include EGFR gene amplification, missense mutations, and deletion/insertion mutations. There are 40 common mutations present in exons 18-21 of the EGFR gene associating with NSCLC incidence. The most common EGFR mutations associated with AD of the lung (90%) are exon 19 deletions and L858R missense mutation in exon 21. Here, we report the results of a study investigating the adequacy of current standards of fine-needle aspiration (FNA) for use in molecular testing of EGFR in NSCLC samples, and for reporting evidence of EGFR mutation in Arabs.

Materials and Methods

Sample collection
Eighteen patients diagnosed with primary NSCLC during 2009-2014 who had FNA cell blocks available were identified. The study was performed according to the guidelines of the local ethics committee which conform to the Helsinki declaration. The samples included 8 primary ADs, and 10 primary NSCLC classified specimens. The cases were selected based on their immunohistochemical status of TTF-1 and p63 positive staining, and availability of FNA cell blocks. Hematoxylin and eosin staining was done on aspirated material in cell blocks of each case and they were reviewed by a pathologist to confirm the presence of tumor cells.

DNA extraction
Genomic DNA was extracted from 10-12 paraffin sections (10 μm thick) from each cell block using Qiagen mini DNA extraction kit (Qiagen, CA, USA) and according to an established protocol with minor modifications. All samples that had a DNA yield less than 10 ng/μl had their two elutions pooled and lyophilized to concentrate the overall yield. The extracted DNA was stored at −20°C until use.

Peptide nucleic acid clamp epidermal growth factor receptor mutation detection
PNA Clamp™ EGFR Mutation Detection Kit (Panagene Inc, Daejeon, Korea) was used to detect 40 most common mutations that are present in exons 18-21 of the EGFR gene. A total of 13 deletions and 13 insertions in exon 19, five insertions in exon 20, and nine missense mutations in exons 18, 20, and 21. All reactions were set up according to manufacturer-recommended protocol. DNA concentration per well was ideally 25 ng/μl. However, DNA yield was low in six samples and a concentration of 12-12.5 ng/μl was used in these cases. Analysis was performed according to manufacturer guidelines and supplemented analysis sheet. In brief, cycle threshold (Ct) values for samples and supplied controls were verified to be within the valid and acceptable ranges provided. ∆Ct-1 values were computed by subtracting sample Ct values from standard Ct values provided. ∆Ct-2 was calculated by subtracting Ct value of samples and Ct value of non-PNA mix control. Results were assessed based on the values of ∆Ct-1 and ∆Ct-2 cross-referenced to manufacturer guidelines [Table 1].

EGFR gene amplification using fluorescent in situ hybridization
EGFR-FISH was performed using Vysis EGFR/CEP7FISH Probe kit (Abbott Laboratories, IL, USA) following the manufacturer’s recommended protocol. Briefly, paraffin sections were deparaffinized in xylene and dehydrated in descending concentrations of ethanol before hydration with water and xylene, and coverslipping with Vysis BrightField Plus medium. Detection of 1 copy of EGFR (green) and 1 copy of CEP7 (red) per cell is identified with either a green or red signal (green only: <2 copies per cell, red only: ≥3 copies per cell, or red and green colocalized: 2 copies per cell). EGF-FISH results were analyzed by a pathologist blinded to clinical data.

Table 1: Criteria for analysis of EGFR mutation genotyping results and EGFR-FISH results

| Classification | EGFR:CEP7 ratio | Scoring criteria |
|----------------|----------------|-----------------|
| Disomy         | ≤2 copies in >90% of cells | ~1 |
| Low trisomy    | ≤2 copies in ≥40% of cells | ~1 |
| High trisomy   | ≤2 copies in ≥40% of cells | ~1 |
| Low polysomy   | ≥4 copies in 10%-40% of cells | ~1 |
| High polysomy  | ≥15 colocalized clusters of EGFR and CEP7 in ≥10% of cells | ~1 |
| Amplified      | ≥15 copies per cell in ≥10% | ≥2 |
|                | ≥4 copies in small cluster in ≥10% | ≥2 |

1EGFR: Epidermal growth factor receptor, PNA: Peptide nucleic acid, EGFR-FISH: EGFR gene amplification using fluorescent in situ hybridization
in absolute ethanol and air-dried. Slides were pretreated in pretreatment solution at 80°C for 12 min followed by protease treatment. Probes were added to processed slides for overnight incubation at 37°C for hybridization. The slides were washed in consecutive wash buffers, air-dried, and counterstained with 4',6-diamidino-2-phenylindole (DAPI). The slides were viewed on a fluorescent microscope with a suitable set filters for spectrum orange (EGFR) and spectrum green (CEP7) signal enumeration. Both green and orange signals are counted in the same cell, and a calculated average number of total green and orange signals per specimen were used to calculate the ratio of the total number of orange to green signals. Fluorescence in situ hybridization (FISH) analysis of EGFR gene amplification (GA) positive specimens was performed according to the established guidelines as shown in Table 1.[10]

**Immunohistochemistry**

Monoclonal mouse antihuman EGFR antibody, clone H-11 (Dako North America Inc., CA, USA) was used at a dilution of 1:50 for immunohistochemical detection of EGFR protein expression in FNA cell blocks’ sections. The immunostaining procedure was performed using Envision Minikit High pH (Dako North America Inc., CA, USA) according to manufacturer protocols. EGFR expression was scored semi-quantitatively on a scale of 0, 1+, 2+, and 3+ based on the brown color intensity staining clearly localized to the cell membranes by an experienced histopathologist. The membranous staining for EGFR was scored using the criteria for Her-2/neu assessment in gastric cancer as follows: 0, no staining of the tumor cells; 1+, faint or barely perceptible membranous staining of the tumor cells; 2+, weak to moderate complete, basolateral, or lateral membranous reactivity in tumor cells; and 3+, strong complete, basolateral, or lateral membranous reactivity in tumor cells.[11] The currently used H-score derived by summing the products of the percentage of positive cells (0%-100%) and the intensity (0-4) for that group, resulting in a final immunohistochemistry (IHC) score ranging 0-400 was tried but was not possible as some biopsies had few cells.[12]

**Results**

NSCLC patients included in this study’s demographics and clinical characteristics are presented in Table 1. There were eight females and 10 males of mixed nationalities. The median age was 55 years (range: 40-84 years). The diagnosis of AD was based on histopathological assessment of distinct patterns inclusive of lepidic growth pattern, acinar pattern, papillary pattern, micropapillary pattern, and solid pattern with mucin. NSCLC was attributed to specimens of nonspecific patterns pertaining to AD or squamous cell carcinoma. There were eight morphologically distinct ADs, and 10 undefined NSCLCs. The size of tumor on radiology was more than 4 cm in most patients (55.5%), and 2–3 cm in other patients. Confirmatory immunohistochemical assay of two cellular markers TTF-1 and p63 were performed to ascertain AD and squamous cell carcinoma diagnoses, respectively. TTF-1 was expressed in 10 out of 18, of which 7 (70%) were morphologically diagnosed ADs. P63 expression was only seen in one case of morphologically diagnosed AD [Table 1].

**EGFR** mutation and expression profile were assayed in all samples [Tables 2 and 3]. EGFR protein was expressed in three samples. However, the intensity was low (one case 1+ and two cases 2+). EGFR mutational analysis revealed five out of the 18 samples as being positive for at least one EGFR mutation. Four confirmed ADs had **EGFR** mutations while one NSCLC that was positive for TTF-1 expression had an **EGFR** mutation. **EGFR** exon 19 deletion was found in one AD expressing **EGFR**, whereas the other two **EGFR** expressing NSCLCs were wild type for **EGFR**. Two E19del and two T790M **EGFR** mutants were detected in different patients. Two patients had double mutations in **EGFR** (patients 12 and 16). However, due to assay limitations it is unknown whether these mutations are in “cis” or “trans.” In total, five different **EGFR** mutations were detected in our NSCLC samples. **EGFR-FISH** revealed 10 out of 18 samples (55.5%) having **EGFR** gene amplification either by gene-specific amplification or chromosomal polysomy. Two of the 10 samples later developed brain metastases, one of which had double mutations in the **EGFR** gene accompanying gene amplification (sample 16) while the other had an **EGFR** protein expression (sample 15).

**Discussion**

Lung cancer incidence contributes to 13% of the estimated 14,090,149 cancer cases worldwide according to the World Health Organization (WHO) statistics (GLOBOCAN, 2008) and is predicted to increase.[13,14] FNA has become the common procedure in clinical diagnosis of lung cancer, and it is the standard specimen collection procedure for lung cancer in Kuwait. One of the major limitations of this procedure is its limited sample size, and disproportionate normal to tumor cell populations, which greatly affects EGFR demonstration by IHC. Several studies have reported successful molecular testing of NSCLC FNA specimens in combination with other morphological and immunohistochemical analyses.[15,16] However, these reports have used additional biopsy material and complex techniques requiring specialized molecular equipment that is not common in a routine molecular diagnostic laboratory. Considering the limitations of FNA samples and the benefits of molecular subtyping of NSCLCs
for treatment options, we have shown that such limitations can be overcome and valuable information can be gained.

**EGFR** mutations occur in 10-23% of ADs, and are very rare in squamous cell carcinomas.\(^{17}\) None of the five Kuwaiti samples had any **EGFR** mutations, whereas five different mutations were evident in five non-Kuwaiti patients. Exon 19 deletions were seen in two cases of Syrian nationality. This mutation was reported to be the most common mutation in the Lebanese population, which is of a closely related ethnic background to Syria, and share a similar environment and lifestyle.\(^{18}\) Exon19del **EGFR** mutations overlap with the start of the kinase domain in the protein and have been established as responsive mutants to TKI.\(^{19}\) In addition, the

| Case no. | Nationality  | Age (year) | Sex | FNA site     | Cytological diagnosis                | Immunohistochemistry |
|----------|--------------|------------|-----|--------------|--------------------------------------|----------------------|
| 1        | Non Kuwaiti  | 64         | Male | CT/right upper | Poorly differentiated NSCLC           | +                    |
| 2        | Kuwaiti      | 75         | Male | CT/right lower | Adenocarcinoma                        | +                    |
| 3        | Non-Kuwaiti  | 51         | Male | CT/left upper  | NSCLC                                | —                    |
| 4        | Kuwaiti      | 59         | Female | CT/right upper | Adenocarcinoma                        | +                    |
| 5        | Syrian       | 40         | Male | CT/right upper | Poorly differentiated NSCLC           | —                    |
| 6        | Syrian       | 47         | Female | CT/right lower | Adenocarcinoma                        | +                    |
| 7        | Syrian       | 46         | Male | CT/right upper | NSCLC                                | —                    |
| 8        | Kuwaiti      | 84         | Male | CT/right upper | NSCLC-possibly adenocarcinoma         | +                    |
| 9        | Egyptian     | 51         | Male | CT/right lower | Poorly differentiated NSCLC           | —                    |
| 10       | Non Kuwaiti  | 54         | Male | CT/right lower | Adenocarcinoma                        | —                    |
| 11       | Syrian       | 63         | Male | CT/left lower  | Adenocarcinoma                        | +                    |
| 12       | Egyptian     | 53         | Female | CT/right upper | NSCLC                                | +                    |
| 13       | Not available| 68         | Male | CT/right upper | NSCLC                                | +                    |
| 14       | Not available| 53         | Female | US/right upper | Adenocarcinoma                        | +                    |
| 15       | Lebanese     | 56         | Female | CT/left upper  | Poorly differentiated NSCLC           | —                    |
| 16       | Non-Kuwaiti  | 51         | Female | CT/right upper | NSCLC-possibly adenocarcinoma         | +                    |
| 17       | Kuwaiti      | 63         | Female | CT/right upper | Adenocarcinoma                        | —                    |
| 18       | Kuwaiti      | 72         | Female | CT/left upper  | Adenocarcinoma                        | —                    |

NSCLC: Nonsmall cell lung carcinoma, FNA: Fine-needle aspiration, CT: Computed tomography, TTF-1: Thyroid transcription factor, p 63: Tumor protein 63 antibody

Table 3: EGFR expression, mutation screen, and gene amplification in aspirates from patients with nonsmall cell lung carcinoma

| Case no. | Age (years) | Sex | Cytological diagnosis | EGFR expression |
|----------|-------------|-----|-----------------------|-----------------|
|          |             |     |                       | IHC | Mutations (Genotyping PCR) | Gene amplifications (FISH) |
| 1        | 64          | Male | Poorly differentiated NSCLC | —   | Wild type | Low polysomy |
| 2        | 75          | Male | Adenocarcinoma         | —   | Wild type | Low polysomy |
| 3        | 51          | Male | NSCLC                 | —   | Wild type | Autofluorescence |
| 4        | 59          | Female | Adenocarcinoma       | —   | Wild type | Disomy |
| 5        | 40          | Male | Poorly differentiated NSCLC | —   | Wild type | Disomy |
| 6        | 47          | Female | Adenocarcinoma       | 2+  | E19del | High polysomy |
| 7        | 46          | Male | NSCLC                | —   | Wild type | Low trisomy |
| 8        | 84          | Male | NSCLC—possibly adenocarcinoma | —   | Wild type | High polysomy |
| 9        | 51          | Male | Poorly differentiated NSCLC | —   | Wild type | No cells |
| 10       | 54          | Male | Adenocarcinoma        | —   | T790M | High polysomy |
| 11       | 63          | Male | Adenocarcinoma        | —   | E19del | High polysomy |
| 12       | 53          | Female | NSCLC                | —   | G719X, T790M | Autofluorescence |
| 13       | 68          | Male | NSCLC                | —   | Wild type | Disomy |
| 14       | 53          | Female | Adenocarcinoma       | 1+  | Wild type | Amplified |
| 15       | 56          | Female | Poorly differentiated NSCLC | 2+  | Wild type | High polysomy |
| 16       | 51          | Female | NSCLC—possibly adenocarcinoma | —   | E20Ins.3dup, L861Q | High polysomy |
| 17       | 63          | Female | Adenocarcinoma       | —   | Undetermined | Low trisomy |
| 18       | 72          | Female | Adenocarcinoma       | —   | Wild type | High Polysomy |

CT: Computed tomography, NSCLC: Nonsmall cell lung carcinoma, EGFR: Epidermal growth factor receptor, IHC: Immunohistochemistry, Genotyping PCR: Genotyping polymerase chain reaction, FISH: Fluorescent in situ hybridization
L861Q mutation in exon 18 is also associated with good response to TKI, and was found in one patient.\textsuperscript{[20]} Other mutations of clinical significance are: Insertion in exon 20, and T790M that overlaps with the middle and end of the kinase domain. Insertions in exon 20 and T790M show poor response to TKI and have a more aggressive clinical course, and are being targeted by an alternative chemotherapeutic agent in a current clinical trial.\textsuperscript{[21-24]} In one patient with exon 20 insertion and a missense mutation in exon 21 brain metastases developed despite chemotherapy, and the mutation was accompanied with EGFR gene amplification and chromosome 7 polysomy in tumor cells. In another sample T790M mutation was found, along with a G719X mutation; the X here denotes any of the following amino acids — A, C, or S.\textsuperscript{[25]} The co-occurrence of two mutations in patients is expected to confound their predicted response to TKI, and would provide a valuable insight into the mechanism of TKI resistance/responsiveness. From a technical perspective, utilizing the PNA clamp assay for mutational screen of small quantities of DNA was successful for FNA specimens, and showed superior sensitivity to low DNA concentration than expected. The procedure was short and run time was 2 h with direct results’ interpretation. However, the assay does not allow novel mutant discovery, which might present targeted gene sequencing as a better assay strategy for EGFR mutational screen. In addition, the assay does not determine the phase of double mutants, or the zygosity of mutations, which might be of research significance.

Estimating EGFR copy number in FNA samples was difficult using FISH technique due to several issues, which include: Few or no cells present, reduced tumor cell population, increased autofluorescence, and increased aneusomy 7, which might be an artefact of uneven planar cell distribution. The use of FNA cell blocks for FISH needs to be optimized for EGFR copy number assessment. In our small sample size 10 NSCLCs had EGFR gene amplification, out of which six were ADs, and EGFR expression was confirmed in three NSCLC samples that had an EGFR gene amplification event. The accurate assessment of EGFR copy number influence on EGFR expression has been previously reported to be dependent on type of antibody used for analysis.\textsuperscript{[26,27]} Four EGFR amplification samples had accompanying EGFR gene mutations; the significance of these findings remains inconclusive due to lack of follow-up data, and the treatment administered. In general, EGFR gene copy number has not been clinically recognized as a marker for NSCLC response to TKI and was performed here for investigative purposes and to determine the efficiency of FNA samples for multiple molecular analyses.\textsuperscript{[28]}

Despite the limitations of our small sample size, it is clear that EGFR is a universal molecular marker for NSCLC, and that FNA material is sufficient for efficient multiple assay analysis. There is a need for comprehensive screening of EGFR mutations in Arab NSCLC patients to determine the exact contribution of EGFR mutations.

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Conflicts of interest
There are no conflicts of interest.

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