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RESEARCH ARTICLE

EPIDERMAL GROWTH FACTOR RECEPTOR MUTATIONS IN LEBANESE NON-SMALL CELL LUNG CANCER PATIENTS

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ABSTRACT

Non Small Cell Lung Cancer (NSCLC) is the major subtype of lung cancers (85%) which is the leading cause of cancer related deaths worldwide. Advances in personalized medicine and in our understanding of the tumor biology along with the identification of special molecular alterations have allowed the development of new treatment approaches for NSCLC, thus providing a marginal improvement in responses and overall survival (OS). Small tyrosine kinase inhibitors (TKIs), which target the tyrosine kinase intracellular domain of the epidermal growth factor receptor (EGFR), have had a remarkable efficacy in treating NSCLC but the responses varied between the patients. This variation is due to the EGFR mutational status. In normal cells, epidermal growth factors (EGF) bind to EGFR and activate several signal transduction pathways which regulate cell differentiation, proliferation, migration, survival, angiogenesis, and apoptosis. In NSCLC, EGFR can be altered either by over-expression or by different mutations. The mutations can occur in exons 18,19,20,21. Clinical studies have shown that if the patient harbors the EGFR mutation, the progression free survival (PFS) is higher than the one with a wild type gene when treated with TKIs. The aim of this diploma work is to perform, an epidemiologic study to determine the prevalence of EGFR mutations in Lebanese NSCLC patients and its correlation with the patient's gender. To process this study, we've analyzed the data provided by the Lebanese National institute of pathology (INP). These data consisted of reports revealing EGFR mutational status in addition to patients personal information of 180 formalin fixed paraffin embedded NSCLC tumor tissues (FFPET). Statistical tests have shown that the EGFR mutations occur in 13.3% of the NSCLC cases in Lebanon, which means that only 13.3% of the patients benefit from the TKI therapy. We've also found that there is no significant correlation between the EGFR mutational status and the patient's gender, and the highest mutational rate was detected in exon 19 (75%), followed by exon 21 (21%).

INTRODUCTION

Non-small-cell lung cancer (NSCLC) is one of the most frequent human malignancies, constituting 85% of all lung tumors (Ferlay *et al.,* 2008; Surveillance Epidemiology, 2012). NSCLC can be divided into several genetic subsets based on the type of the activating mutation that the patient harbors, mainly affecting the treatment algorithm using specific inhibitors. The two main alterations involving the epidermal growth factor receptor (EGFR) are the protein over-expression which is observed in up to 62% of NSCLC (Rowinsky, 2004; Gazdar and Minna, 2008), and the EGFR somatic mutations, most of which are activating mutations, affecting hotspots within exons that code for the tyrosine kinase domain (exons 18-21), leading to a ligand independent constitutive activation of the tyrosine kinase domain therefore causing uncontrollable cell proliferation and survival. These mutations can be found in 10-40% of NSCLC patients (Noriko Gotoh, 2001), mostly in adenocarcinomas, with the higher frequency observed in female Asian patients (D'Angelo, 2011). About 50% of mutated patients harbor in-frame deletions in exon 19, (around codons 746 to 750) and 35-45% show the substitution of leucine 858 by an arginine in the exon 21 (L858R). The remaining mutants are insertions in exon 20 (5%) and uncommon substitutions spanning exons from 18 to 21, such as G719X in exon (Mitsudomi and Yatabe, 2007). These specific mutations are related to a higher sensitivity to the tyrosine kinase inhibitors (TKIs) Erlotinib and Gefitinib. The centrality of EGFR signaling has led to intensive efforts to

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design inhibiting therapies. Initial efforts were used to block the ligand-receptor interaction with monoclonal antibodies (Erbitux® or Cetuximab), however new small molecules that target the TK activity of EGFR (Iressa® or Gefitinib and Tarceva® or Erlotinib) by blocking the Adenosine triphosphate (ATP) binding site (upon which signaling depends) have displayed remarkable efficacy in NSCLC patients with mutations in the EGFR gene (Hirsch *et al.,* 2003 ; Sharma *et al.,* 2007). Curiously, the TKIs were designed before the elucidation, in 2004 by three American groups, of the EGFR mutation (Pao *et al.,* 2004; Lynch *et al.,* 2004; Oxnard *et al.,* 2001). The small subset of metastatic NSCLC patients who had responded to TKI therapy prompted the search for an explanation, culminating in discovery of the mutations. It soon became clear that almost all the dramatic responses had occurred in patients whose cancers harbored one of these activating EGFR mutations; however, EGFR-TKIs can also, with a lower rate, benefit patients without those mutations: that is, the EGFR "wild-type" (EGFR WT) patients, whose cancers are presumably driven by up-regulated signaling (from over-expression of the normal protein, for instance). Unfortunately, approximately 50% of NSCLC patients, who respond initially to reversible first generation EGFR-TKIs, eventually develop resistance by acquiring a second recurrent missense mutation in the EGFR kinase domain (Oxnard *et al.,* 2001). This study was launched in order to determine the prevalence of EGFR mutations in Lebanese NSCLC patients and compare it to the worldwide prevalence of these mutations (10-40% of NSCLC) as well as analyzing its correlation with the patient's gender.

MATERIALS AND METHODS

Patients and tissues

180 FFPET specimens from both male and female NSCLC patients were included in this retrospective study. These samples were processed at the molecular biology department of the INP using Food & Drug Administration (FDA) approved kits in screening for EGFR mutations. The patient's ages varied between 41 and 86 years old. All the samples, after microscopic assessment H&E stained slides by a licensed pathologist, displayed a content in tumor cells which was greater than 10% (therefore, a macro-dissection was not required prior to deparaffinization).

Work flow

Each sample, in order to achieve the Cobas® EGFR- mutation test properly, must follow a certain procedure described below.

H&E staining

Upon a test requisition by the treating physician, a specimen that belongs to the patient is sent to the lab for histo-pathological and molecular analysis. The biopsy is processed in a specialized tissue processor for the fixation, hardening and preservation of the tissue and then embedded into a paraffin block. Using a microtome the block will be micro-dissected and mounted on glass slides. The need for macro-dissection must be determined prior to deparaffinization. For each FFPET block section, a pathologist will perform a microscopic assessment of an Hematoxylin & Eosin (H&E) stained slide and will document the percentage of tumor cells content. The staining method involves application of Hemalum (Complex formed from aluminium ions and oxidized haematoxylin) that colors the nuclei of cells with blue. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of Eosin Y, which colors eosinophilic structures (cytoplasm and cellular membrane) in various shades of red, pink and orange. A macro-dissection is required for DNA isolation, in case the sample displayed less than 10% content in tumor cells, which is not the case in this study.

Deparaffinization of FFPET sections

In order to proceed, FFPET sections must be deparaffinized by adding Xylene and then Ethanol to each sample. After centrifugation, the supernatant is discarded without disturbing the pellet that will be left to dry at 56° for 15 mins.

Genomic DNA isolation

Deparaffinized FFPET sections are processed and genomic DNA is isolated using the cobas® DNA Sample Preparation Kit, a generic manual specimen preparation based on the binding of nucleic acid to glass fibers. The pellet is resuspended in Desoxyribonucleic Acid Tissue Lysis Buffer (DNA TLB), and Reconstituted Proteinase K (RPK) will be added. A negative control (NEG CT) will be processed simultaneously with the samples. It's prepared by combining DNA TLB and PK solution in a Safe-Lock micro-centrifuge tube. The negative control should be processed using the same procedure as the samples. The tubes containing DNA/TLB/PK mixture and the NEG CT are placed in the 56°C dry heating plate for 60 min and then in the 90°C heating plate for another 60 min with a simple vortex step following each incubation. Subsequently, DNA Paraffin Binding Buffer (DNA PBB) and Isopropanol are added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the genomic DNA is bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed twice by the Washing Buffer I (WBI) and Washing Buffer II (WBII) and then eluted with the DNA Elution Buffer (DNA EB).

DNA quantification

Extracted DNA should be subject to spectrophotometric evaluation using a Nano Drop 2000® prior to the amplification step. This step will determine the DNA concentration in 2 µl of the extracted solution which is an important determinant for PCR parameters; it will also give the ratio of absorbance (optical density) 260/280 (Fig. 1). This ratio is used to assess the purity of DNA. A ratio of ~ 1.8 is generally accepted as pure DNA. If the ratio is appreciably lower, it may indicate the contamination with proteins (since proteins have a maximum absorbance at 280 nm) or other contaminant reagents associated with the extraction protocol that absorb strongly at or near 280 nm. However, a ratio higher than 2 indicates other impurities that absorb strongly at 260 nm (such as compounds with carboxylic acid groups). An optical density (OD) of 1 corresponds to a 50µg/µl of double stranded DNA. Concentrations of the extracted DNA must be ≥ 2 ng/µL to perform the Cobas® EGFR-Mutation test.

PCR amplification

After being spectrophotometrically quantified, the target DNA is then amplified and detected through PCR analysis on the Cobas z 480 analyzer using the amplification and detection reagents provided in the Cobas® EGFR Mutation Test kit. Three amplification/detection reactions are run per specimen, each using 25 µL of a 2 ng/µL diluted solution of DNA stock, so for each specimen, the volume of DNA stock and the volume of DNA Specimen Diluent (DNA SD) needed to prepare a 2 ng/µL solution must be calculated. The preparation of diluted solution of specimens and negative control is followed by the preparation of Working Master Mixes (MMX-1, MMX-2 and MMX-3):

- The volume of EGFR MMX-1 or EGFR MMX-2 or EGFR MMX-3 required for each working MMX is calculated using the following formula:

Volume of EGFR MMX-1 or EGFR MMX-2 or EGFR MMX-3 required = (Number of Specimens + 2 Controls +1) x 20 µL

- The volume of Magnesium Acetate (MGAC) required for each working MMX is calculated using the following formula:

Volume of MGAC required = (Number of Specimens + 2 Controls +1) x 5 µL

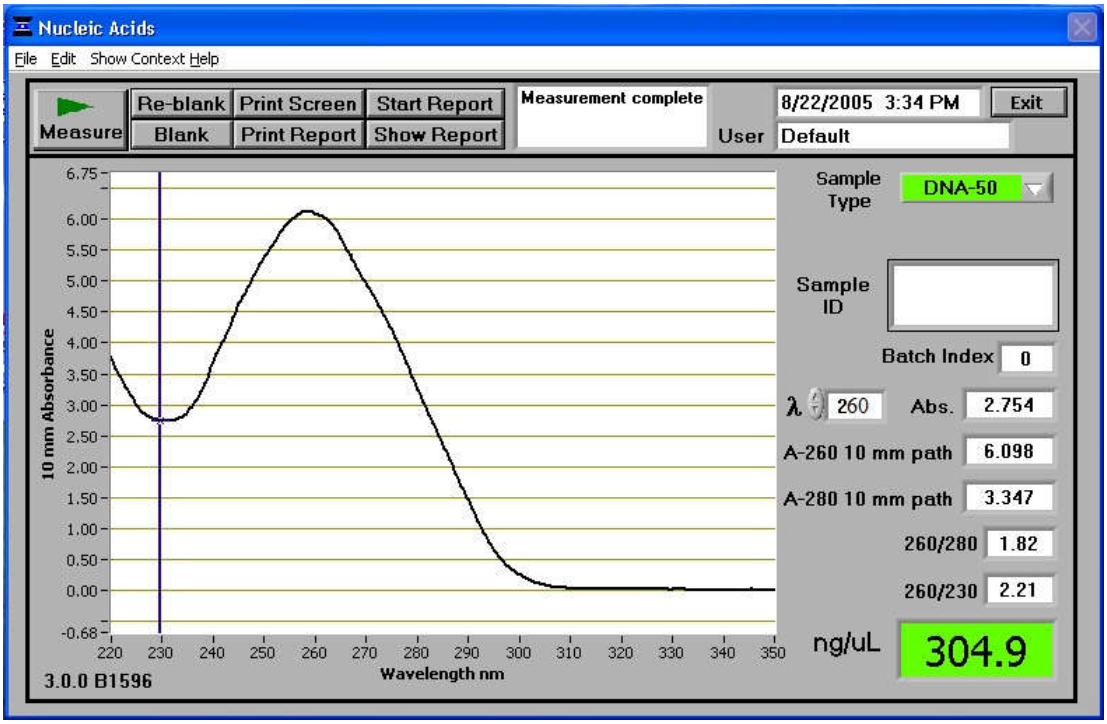

Figure 1. Example of the NanoDrop 2000® Result Display

Row / Column	1	2	3	4	5	6	7	8	9	10	11	12
A	MUT MMX-1	MUT MMX-2	MUT MMX-3	S7 MMX-1	S7 MMX-2	S7 MMX-3	S15 MMX-1	S15 MMX-2	S15 MMX-3	S23 MMX-1	S23 MMX-2	S23 MMX-3
B	NEG MMX-1	NEG MMX-2	NEG MMX-3	S8 MMX-1	S8 MMX-2	S8 MMX-3	S16 MMX-1	S16 MMX-2	S16 MMX-3	S24 MMX-1	S24 MMX-2	S24 MMX-3
C	S1 MMX-1	S1 MMX-2	S1 MMX-3	S9 MMX-1	S9 MMX-2	S9 MMX-3	S17 MMX-1	S17 MMX-2	S17 MMX-3	S25 MMX-1	S25 MMX-2	S25 MMX-3
D	S2 MMX-1	S2 MMX-2	S2 MMX-3	S10 MMX-1	S10 MMX-2	S10 MMX-3	S18 MMX-1	S18 MMX-2	S18 MMX-3	S26 MMX-1	S26 MMX-2	S26 MMX-3
E	S3 MMX-1	S3 MMX-2	S3 MMX-3	S11 MMX-1	S11 MMX-2	S11 MMX-3	S19 MMX-1	S19 MMX-2	S19 MMX-3	S27 MMX-1	S27 MMX-2	S27 MMX-3
F	S4 MMX-1	S4 MMX-2	S4 MMX-3	S12 MMX-1	S12 MMX-2	S12 MMX-3	S20 MMX-1	S20 MMX-2	S20 MMX-3	S28 MMX-1	S28 MMX-2	S28 MMX-3
G	S5 MMX-1	S5 MMX-2	S5 MMX-3	S13 MMX-1	S13 MMX-2	S13 MMX-3	S21 MMX-1	S21 MMX-2	S21 MMX-3	S29 MMX-1	S29 MMX-2	S29 MMX-3
H	S6 MMX-1	S6 MMX-2	S6 MMX-3	S14 MMX-1	S14 MMX-2	S14 MMX-3	S22 MMX-1	S22 MMX-2	S22 MMX-3	S30 MMX-1	S30 MMX-2	S30 MMX-3

Where: NEG = Negative Control, MUT = Mutant Control, S# = sample ID, and MMX-# corresponds to Master Mix Reagent 1, 2, or 3.

Table 1. Sample Plate layout for the cobas® EGFR Mutation Test

To prepare the plate (*Use only cobas® 4800 System Microwell Plate (AD-Plate) and Sealing Foil)*: 25 µL of working MMX-1, MMX-2, MMX-3 are added to the AD-Plate wells, followed by 25 µL of EGFR Mutant Control (EGFR MC), 25 µL NEG CT, and 25 µL diluted specimen DNA as shown in the following sample plate layout (Table 1). After being covered with sealing foil, the plate is placed in the Cobas z480 Analyzer so that the amplification and detection of target DNA through PCR amplification begins.

Data analysis

One set of Cobas® EGFR MC and NEG CT for working MMX-1, working MMX-2, and working MMX-3 are included in each run of up to 30 specimens. A run is valid if the EGFR MC wells, and the NEG CT wells are valid. If the EGFR MC or NEG CT for working MMX-1 or working MMX-2 or working MMX-3 is invalid, the entire run is invalid and must be repeated. For a valid run, specimen results are interpreted as shown in Table 2. The obtained results, corresponding to the 180 FFPET specimens, were collected from the database and classified according to the mutation result (detected or not detected), the gender, and the type of mutation if it existed. Statistical calculations were performed using IBM SPSS statistics 20 software:

Frequencies were calculated to determine the prevalence of detected EGFR mutations and the rates of each mutation among all specimens and among female and male patients. Chi-square test was performed to measure the significance of the difference between the calculated rate of detected EGFR mutations and the rate worldwide (10-40%). Cross-tabs analysis and Chi-square test to measure the correlation between EGFR mutational status and the patient's gender.

RESULTS

Using the frequency procedure, the following statistics and graphs describe the distribution of the samples and the rates of individuals harboring an EGFR mutation and of those who aren't (Table.3, Fig. 2). To compare these results to the known and common hypothesis which state that the prevalence of EGFR mutations in unselected cases of NSCLC is approximately 10%, a chi-square test is performed: The χ^2_c calculated is 0.4356 and the χ^2_t is 3.841 (considering α=0.05). Another frequency procedure was executed, to describe the prevalence of the different types of mutations within the samples that have already displayed an EGFR mutation (Table 4, Fig. 3). To measure the correlation between EGFR mutational status and the patient's gender, a cross-tab analysis and chi-square test were performed (Table.5, Fig. 4). The p-value obtained by the chi-square test to evaluate the significance of the results and thus check whether the 2 variables correlate is 0.059. Among males, frequency distribution was analyzed, to describe the prevalence of the different types of mutations within the samples harboring an EGFR mutation (Table.6, Fig. 5). The same procedure was performed among females (Table 7, Fig. 6).

Table 2. Result Interpretation of cobas® EGFR-Mutation Test

Test Result	Mutation Result	Interpretation
Mutation Detected	Exon 19 deletion S768I L858R T790M G719X (G719A, G719C, G719S) Exon 20 Insertion (More than one mutation may be present)	Mutation detected in specified targeted EGFR region.
Mutation Not Detected	N/A	Mutation not detected in targeted EGFR regions.
Invalid	N/A	Specimen result is invalid. Repeat the testing of specimens starting from "Dilution Calculation of Specimen DNA Stock" and "Specimen Dilution" procedures in the "AMPLIFICATION and DETECTION" section.
Failed	N/A	Failed run due to hardware or software failure.

Table 3. EGFR mutational rate in Lebanon 2012-2013

		Frequency	Percent	Valid Percent	Cumulative Percent
Mutation	Not detected	130	86.7	86.7	86.7
	Detected	20	13.3	13.3	100.0
	Total	150	100.0	100.0	

Table 4. Mutation type distribution

		Frequency	Percent	Valid Percent	Cumulative Percent
Mutation	Exon18: G719X	1	5.0	5.0	5.0
	Exon19- Deletion	15	75.0	75.0	80.0
	Exon20- Insertion	1	5.0	5.0	85.0
	Exon21- L858R	3	15.0	15.0	100.0
	Total	20	100.0	100.0	

Table 5. Gender*EGFR mutational status: cross-tabulation test results

% of Total			
	Mutation		Total
	Not etected	Detected	
Male	54.0%	5.3%	59.3%
Female	32.7%	8.0%	40.7%
Total	86.7%	13.3%	100.0%

Table 6. Distribution of male patient's by mutation type

% of Total			
	Mutation		Total
	Not detected	Detected	
Male	54.0%	5.3%	59.3%
Female	32.7%	8.0%	40.7%
Total	86.7%	13.3%	100.0%

Table 7. Distribution of female patient's by mutation type

	Frequency	Percent	Valid Percent	Cumulative Percent
Exon 19	5	62.5	62.5	62.5
Exon 20	1	12.5	12.5	75.0
Exon 21	2	25.0	25.0	100.00
Total	8	100.0	100.0	

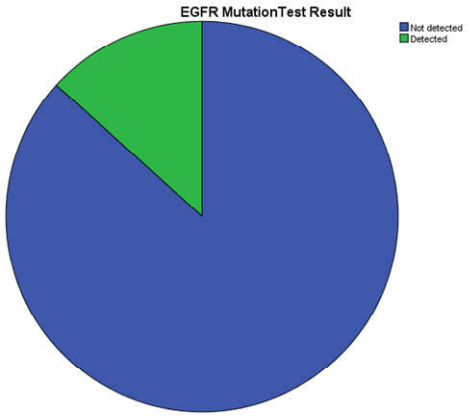

Figure 2. Percentage of observed EGFR mutations in Lebanon 2012-2013

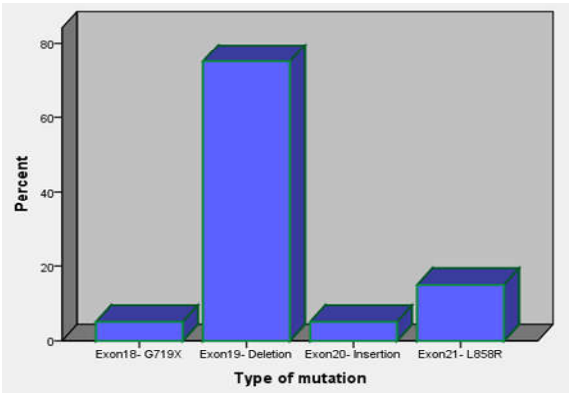

Figure 3. Mutation type distribution

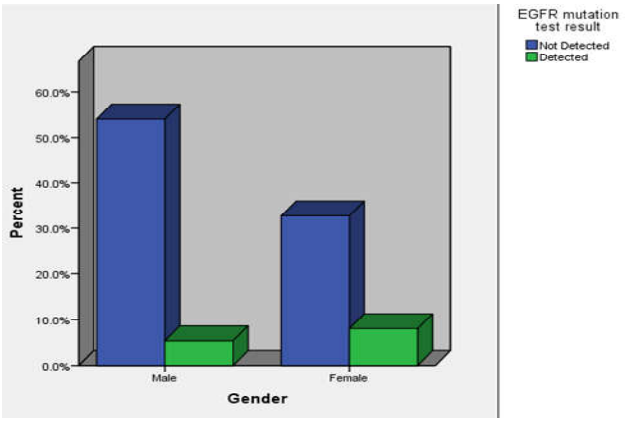

Figure 4. Gender*EGFR mutational status test results in %

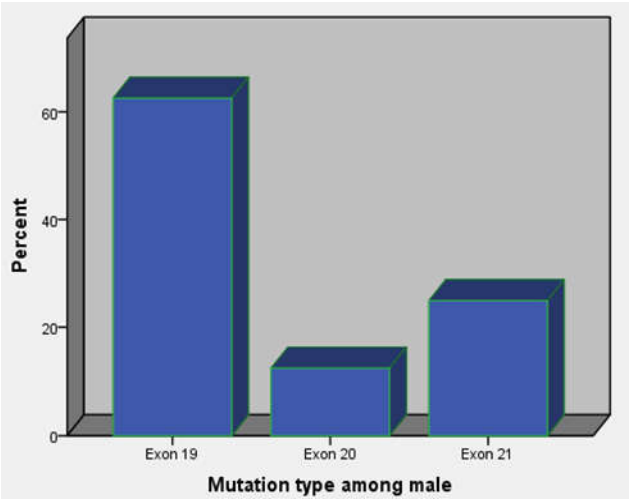

Figure 5. Distribution of male patient's by mutation type

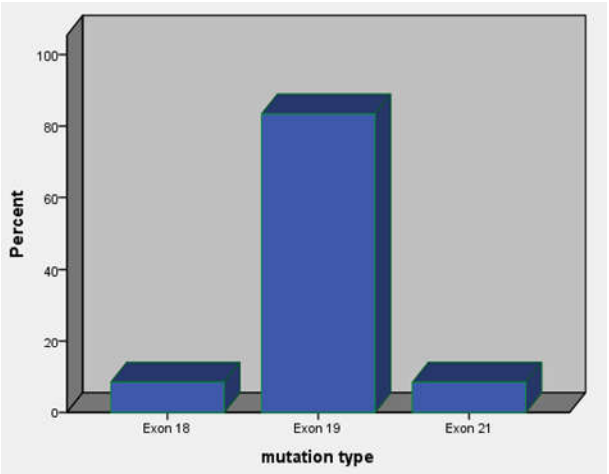

Figure 6. Distribution of female patient's by mutation type

DISCUSSION

The descriptive study resulted in 13.3% of mutated EGFR detected among the studied samples and 86.7% of wild type EGFR gene (Table 3). This prevalence seems to be higher than the common and known prevalence (10%), but the statistical significance test proved this difference to be non significant so the prevalence of the EGFR mutation in Lebanon is the same as in other demographic zones. Among the mutated EGFR cases, the deletion in exon 19 scored the highest rate (75%) followed by the L858R mutation in exon 21 (15%) and 5 % to each of the mutations in exon 18 and 20 (Table 4). Studying the relation between the EGFR mutation and the gender, 8% of the Females diagnosed with NSCLC harbor an EGFR mutation versus 5.3% of the Males (Table 5). The calculated p-value which is higher than 0.05, indicates that the 2 variables are independent and the difference between the prevalence of EGFR mutations among males and the one among females is non significant. But what differs between the 2 is the type of EGFR mutation; though the most frequent mutation occurs in the exon 19 among both gender, but the difference remains in the other types of mutations. Among females, the mutation in the exon 18 can be observed while it is not detected among males. And vice-versa for the mutation in the exon 20, that is observed among males only. These results indicate that only 13.3% of the lebanese patients diagnosed with NSCLC, benefit

the anti-EGFR TKIs therapies. The patients harboring a wild type EGFR gene will further process a test for Anaplastic Lymphoma Kinase (ALK) rearrangements.

ALK is a member of the insulin superfamily of receptor tyrosine kinases which is located on chromosome 2 (chr 2p23). Several chromosomal rearrangements involving the ALK gene occur in a variety of malignancies including NSCLC. It was shown that a small inversion within chr 2p result in the formation of a fusion gene comprising portions of Echinoderm Microtubule associated protein-Like 4 (EML4) gene and the ALK gene . The fusion gene results in constitutive ALK kinase activation serving as a potent oncogenic driver with transforming ability (Soda *et al.,* 20047). The EML4-ALK fusion transcript could be detected in 6% of NSCLC patients (Shaw *et al.,* 2009). In these tumors, ALK is the determinant of critical growth pathway resulting in the activation of: PI3K/AKT and MAPK/ERK pathway (Kwak *et al.,* 2010). EML4-ALK positive patients are treated with ALK TKI (Crizotinib) resulting in a 57% RR, stable disease in 33% of the patients, 10 months PFS, 74% of 1 year OS and 54% of 2 year OS as clinical studies show (Shaw *et al.,* 2011). As in the case of EGFR therapy, the benefit of crizotinib therapy is limited by the development of an acquired resistance (Choi *et al.,* 2010). In the case of EML4-ALK fusion absence, several other genetic alterations, which can occur in NSCLC but with lower prevalence, can be screened for in order to find an effective treatment i.e. BRAF mutations (Paik *et al.,* 2011). Thus, by targeting the 'driver genetic alteration' of each specific molecularly defined subset, the greater improvements in outcome will be seen. These improvements have a short term impact because of the acquired resistance. More potent and/or irreversible inhibitors capable of blocking mutated targets can be used to overcome acquired resistance as well as combined therapies by activation of parallel or downstream pathways.

Conclusion

Clinical data indicate that approximately 10% of patients with advanced NSCLC tumors harbor activating mutations in EGFR, which occur most commonly as deletions in exon 19 or as the L858R point mutation in exon 21 and are independent of the patient's gender. These patients exhibit a high response rate and prolonged progression-free survival (PFS) when treated with anti-EGFR TKIs, both in first- and second-line therapy.
Other less common EGFR mutations, such as G719X substitutions in exon 18 and the L861Q point mutation in exon 21 also predict for responsiveness to anti-EGFR therapies.
Median survival in patients with EGFR-mutant NSCLC treated with anti-EGFR TKIs is now approximately 2 years. Current clinical guidelines recommend considering targeted anti-EGFR TKI therapy as first-line therapy in advanced EGFR-mutant NSCLC. Most NSCLC patients with mutant EGFR eventually develop progressive disease when treated with anti-EGFR TKIs. In approximately half of these progressive cases, the emergence of secondary resistance mutations in the EGFR gene, most often the T790M mutation in exon 20 can be identified (Engelman *et al,* 2008). In some patients, these secondary resistance mutations can be detected in their tumors prior to anti-EGFR TKI therapy. These patients appear to have significantly shorter PFS than patients whose tumors lack these secondary mutations. This acquired secondary resistance to TKI targeted therapy in NSCLC patients with mutant EGFR, represents a valuable model for considering alternative strategies in order to overcome different types of cellular resistance mechanisms. 3 basic approaches for overcoming resistance to targeted anti-EGFR therapies are now being developed and taken into consideration: intensification of EGFR inhibition, combination of EGFR inhibitors with other targeted therapies, and switching to anti-cancer therapies involving alternative pathways (Geoffrey *et al.,* 2012).

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