Analysis of Genetic Alterations in Tunisian Patients with Lung Adenocarcinoma

Dhoa Dhieb, Imen Belguith, Laura Capelli, Elisa Chiadini, Matteo Canale, Sara Bravaccini, Ilhem Yangui, Ons Boudawara, Rachid Jlidi, Tahya Boudawara, Daniele Calistri, Leila Ammar Keskes, and Paola Ulivi.*

1 Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, 47014 Meldola, Italy; dhoha.dhieb@gmail.com (D.D.); laura.capelli@irst.emr.it (L.C.); elisa.chiadini@IRST.emr.it (E.C.); matteo.canale@IRST.emr.it (M.C.); sara.bravaccini@IRST.emr.it (S.B.); daniele.calistri@IRST.emr.it (D.C.)
2 Laboratory of Human Molecular Genetics, Faculty of Medicine of Sfax, University of Sfax, Sfax 3029, Tunisia; mayno87.ib@gmail.com (I.B.); ammarkeskesl@gmail.com (L.A.K.)
3 Department of Respiratory and Sleep Diseases, CHU Hedi Chaker, Sfax 3029, Tunisia; ilhem.bouazizyangui@gmail.com
4 Department of Pathology, CHU Habib Bourguiba, Sfax 3029, Tunisia; annoussa1988@hotmail.com (O.B.); tahya.sellami@RNS.TN (T.B.)
5 Laboratory of Anatomic Pathology, Sfax 3000, Tunisia; rachid.jlidi@gmail.com

* Correspondence: paola.ulivi@irst.emr.it; Tel.: +39-0543-739980

Received: 27 March 2019; Accepted: 26 May 2019; Published: 28 May 2019

Abstract: The identification of the mutations that drive lung cancer have furnished new targets for the treatment of non-small cell lung cancer (NSCLC) and led to the development of targeted therapies such as tyrosine kinase inhibitors that are used to combat the molecular changes promoting cancer progression. Furthermore, biomarkers identified from gene analysis can be used to detect early lung cancer, determine patient prognosis, and monitor response to therapy. In the present study we analyzed the molecular profile of seventy-three Tunisian patients with lung adenocarcinoma (LAD). Mutational analyses for EGFR and KRAS were performed using direct sequencing, immunohistochemistry or MassARRAY. Anaplastic lymphoma kinase (ALK) rearrangement was evaluated by immunohistochemistry using the D5F3 clone, and p53 expression was also assessed. The median age of patients at diagnosis was 61 years (range 23–82 years). Using different methodologies, EGFR mutations were found in 5.47% of patients and only exon 19 deletions “E746-A750 del” were detected. KRAS mutations were present in 9.58% of cases, while only one patient was ALK-positive. Moreover, abnormal immunostaining of p53 was detected in 56.16% of patients. In conclusion, the detected rates of EGFR and KRAS mutation and ALK rearrangement were lower than those found in European and Asian countries, whereas, abnormal p53 expression was slightly more frequent. Furthermore, given the small sample size of this study, a more comprehensive analysis of this patient set is warranted.

Keywords: molecular profile; EGFR; KRAS; ALK; p53; lung adenocarcinoma

1. Introduction

In the past few years the discovery of driver mutations has led to improvements in targeted therapies for lung adenocarcinoma (LAD) [1]. Epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) alterations are the major actionable genetic alterations that are treatable in LAD [2]. EGFR mutations are present in around 15% of patients in Western countries and in 50% of Asians [3,4]. These driver mutations are most frequently found in Asian women and in those who
Cells 2019, 8, 514

have never smoked [5,6]. They have been shown to be predictive of response to first-line treatment with specific tyrosine kinase inhibitors (TKIs) such as gefitinib, erlotinib, and afatinib [7,8], which have led to improved clinical outcomes as compared with standard chemotherapy [9]. ALK rearrangements, a targetable genetic change occurring in around 5% of patients with LAD [10,11], are more frequent in younger individuals who are light or never smokers [12,13]. The above mutations are particularly sensitive to targeted therapies such as crizotinib, alectinib, and ceritinib [14,15]. Screening for EGFR and ALK alterations is now mandatory to determine the appropriate treatment for patients with LAD [4] and should thus be standard practice in the diagnostic workup. In addition, Kirsten rat sarcoma viral oncogene homolog (KRAS) testing is now performed more frequently in LAD. Some studies have reported that KRAS mutations are more prevalent in women, while others have found no differences with regard to gender [16,17]. Although alterations in EGFR, KRAS, and ALK are considered to be mutually exclusive [12,18], there is also evidence that these mutations may overlap [19,20]. On the other hand, tumor-suppressor gene 53 (TP53), which encodes for a protein (p53) regulating cell-cycle arrest, senescence, and apoptosis, is one of the most commonly altered genes in lung cancer. It can thus be hypothesized that loss of p53 function through mutation leads to unchecked proliferation, tumor growth, and therapeutic resistance [21,22]. TP53 gene mutations and epigenetic alterations can be detected by immunohistochemistry (IHC) and frequently result in an accumulation of abnormal protein within tumor cells [23]. The incidence of TP53 mutations in LAD is ~50% according to recent studies and is higher in smokers [24]. International guidelines also recommend that other novel molecular targets such as BRAF, NRAS, PIK3CA, ALK, ERBB2, DDR2, RET, and MAP2K1 variants be analyzed before starting treatment with palliative intent for LAD [25]. The molecular profiling of lung cancer is still a fairly infrequent practice in Tunisia and chemotherapy remains the primary treatment for non-small cell lung cancer (NSCLC). This is in strong contrast to European, Asiatic, and American countries where molecular profiling is well established and different sequencing approaches have been validated [26–28]. Evaluating the mutation status of lung cancer patients provides valuable information that can help to identify the optimal treatment regimen for patients. In the present study we used different methodologies to analyze the molecular profile of a representative set of Tunisian patients with LAD. We also underlined the importance of different sequencing approaches (MassARRAY and Sanger) and the usefulness of IHC in the molecular profiling of LAD.

2. Materials and Methods

2.1. Patients and Tissue Samples

Seventy-three formalin-fixed, paraffin-embedded (FFPE) tissue samples (39 lung biopsies and 34 lung resections) from Tunisian patients with LAD at the Department of Pathology in Habib Bourguiba Hospital in Sfax, Tunisia were analyzed. Hematoxylin- and eosin-stained slides were prepared for each sample and reviewed by 2 experienced pathologists to identify the areas of highest tumor density (i.e., with at least 80% tumor content). Histologic classification was made according to the 2015 WHO criteria [29]. Patients with mixed histology and a coexisting non-pulmonary malignancy were excluded. Clinical and pathological data were obtained from medical records and centrally reviewed for the purposes of the study (Table 1). The overall case series was analyzed using different methodologies (Figure 1).
Figure 1. Flow chart of the analytical methods used.

Table 1. Clinical pathological characteristics of patients (n = 73).

|                          | No. (%) |
|--------------------------|---------|
| All patients             | 73 (100)|
| Age, years               | 73      |
| ≤ 61                     | 36 (49.31)|
| > 61                     | 37 (50.68)|
| Gender                   | 73      |
| Male                     | 61 (83.56)|
| Female                   | 12 (16.43)|
| Tumor stage              | 53      |
| I–II                     | 20 (37.73)|
| III–IV                   | 33 (62.26)|
| Tumor size               | 44      |
| T1–T2                    | 21 (47.72)|
| T3–T4                    | 23 (52.27)|
| Lymph node (N) involvement| 44     |
| N0–N1                    | 27 (61.36)|
| N2–N3                    | 17 (38.63)|
| Metastasis               | 52      |
| M0                       | 33 (63.46)|
| M1                       | 19 (36.53)|
| Smoking status           | 59      |
| Non-smoker               | 14 (23.72)|
| Smokers                  | 45 (76.27)|

2.2. Molecular Analysis

2.2.1. DNA Extraction

Macrodissection was performed using the tip of a blade to scrape off the selected tumor areas on 5-µm unstained slides based on the tumor area selected in hematoxylin- and eosin-stained slides. Cells were lysed in 50 mM of KCl, 10 mM of Tris-HCl pH 8.0, 2.5 mM of MgCl2 and Tween-20 0.45% supplemented by proteinase K at a concentration of 1.25 mg/mL, overnight at 56 °C. Proteinase K was inactivated at 95 °C for 10 min, after which samples were centrifuged twice to eliminate debris. DNA was purified using QIAamp DNA micro kit (Qiagen, Hilden, Germany) following the cleanup
of genomic DNA protocol. After assessing the quantity and quality of the DNA by Nanodrop-2000 (Thermo Fisher Scientific, Monza, Italy), it was stored at −20 °C for further testing.

2.2.2. Mutation Analysis

Exon 2 of KRAS and exons 18–21 of EGFR were amplified in 53 of the 73 cases by polymerase chain reaction (PCR) assay using the primers indicated in Table 2. PCR product was purified using exonuclease and then submitted to sequencing using an ABI PRISM 3100-Avant automated DNA sequencer with the BigDye Terminator Cycle Sequencing reaction kit v1.1. (catalog number 4337450, Thermo Fisher Scientific, Monza, Italy). Each exon was sequenced on both strands, amplified, and then sequenced again on both strands to eliminate the risk of PCR artifacts. KRAS and EGFR, together with BRAF, NRAS, PIK3CA, ALK, ERBB2, DDR2, RET, and MAP2K1 gene mutations were also analyzed by MassARRAY platform (Sequenom, San Diego, CA, USA) using Myriapod Lung Status CE-IVD kit (Diatech Pharmacogenetics, Jesi, Italy) according to the manufacturer’s instructions. The MassARRAY (Sequenom) testing was carried out on 20 cases, and the selection of the methodology to be used was done on the basis of the amount of tumor tissue available.

2.3. Immunohistochemistry

2.3.1. EGFR Mutation-Specific Immunohistochemistry

Immunohistochemical staining was performed on 24 cases. We used EGFR E746-A750del (catalog number 2085, Cell Signaling Technologies (CST), Danvers, MA, USA) and EGFR L858R (catalog number 3197, Cell Signaling Technologies (CST)) as primary antibodies that were manually applied to the slides. The technique was carried out on a Benchmark® GX (Ventana Medical Systems, Tucson, AZ, USA) automated stainer. Immunoreactivity was revealed with UltraView Universal DAB detection kit (Ventana Medical Systems) and slides were counterstained with hematoxylin. Positive and negative controls were used. Each slide was examined and scored independently by two pathologists (Table 3) [30]. A specimen was considered positive in the presence of nuclear and/or cytoplasmic immunostaining for EGFR in tumor cells. Any discordance was resolved by consensus after a joint review using a multihead microscope.

2.3.2. ALK Expression Analysis

Immunohistochemistry was performed on all cases with a monoclonal rabbit ALK antibody (D5F3, Ventana Medical Systems) using an OptiView DAB IHC Detection Kit and an OptiView Amplification Kit. The technique was fully automated on Benchmark GX according to the manufacturer’s recommendations. A sample was classified as positive if strong granular cytoplasmic staining was present in any percentage of tumor cells and negative if the tumor did not show immunoreactivity or if there was only weak or moderate cytoplasmic staining.
Table 2. Primer sequences.

| Forward Primer | Reverse Primer | Annealing Temperature (°C) | Product Size (bp) |
|----------------|----------------|-----------------------------|-------------------|
| KRAS exon 2    | GACTGAATATAAActTGTGGTAGTTG | TTGGATCATATTCGTCCACAA     | 60               | 101 |
| EGFR exon 18   | TCCAATGACGTGCAAGTG       | TCCCCAACACTCAGTGAACAAA    | 60               | 397 |
| EGFR exon 19   | CGTCTCTCTCTCTCTGTC       | GACATGAGAAAGGTGGGC        | 60               | 190 |
| EGFR exon 20   | CATTCATGGCTCTACACCTG     | CATATCCCCATGGCAAACTC      | 60               | 377 |
| EGFR exon 21   | GCTCAGACCTTGCAATG       | CATCCTCCCCTGCACTG         | 60               | 348 |

Table 3. Epidermal growth factor receptor (EGFR) immunostaining score.

| Staining Intensity | No Staining | Weak (1) | Moderate (2) | Strong (3) | Very Strong (4) |
|--------------------|-------------|----------|--------------|------------|-----------------|
| Proportion of positive tumor cells | 0% to 100% |          |              |            |                 |

Immunostaining score = positive cell proportion score multiplied by staining intensity score

\[ H = 1 \times (\% \text{cells 1+}) + 2 \times (\% \text{cells 2+}) + 3 \times (\% \text{cells 3+}) + 4 \times (\% \text{cells 4+}) \]

| Degree of immunostaining | Negative or low | High |
|--------------------------|-----------------|------|
| Proportion of positive tumor cells | 0% to 100% | 200–400 |
2.3.3. p53 Expression Analysis

Immunohistochemical staining was performed using p53 monoclonal antibodies (Clone: DO-7, code: M7001, dilution, 1/50, Dako). Positive and negative controls were used to validate the reactions. p53 immunostaining was assessed semiquantitatively on the basis of staining intensity, and proportion of positive tumor cells (Table 4).

2.4. Compliance with Ethical Standards and Informed Consent

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Sfax University, Tunisia, (CPP SUD N° 34/2016, 10/2016). The need for informed consent was waived because of the retrospective nature of the study.

3. Results

3.1. Patient Characteristics

Of the 73 patients with LAD enrolled in the present study, 12 (16.43%) were females and 61 (83.56%) were males. The median age was 61 years (range 23–82 years). The FFPE samples were adequate for final diagnosis and molecular analysis in all cases. The TNM classification was available for 53 patients, 33 (62.26%) classified with stage III or IV disease and 20 (37.73%) with stage I or II, and 36.53% (19/52) of patients had metastases. Smoking status was known for 59 patients of whom 45 patients were smokers (76.27%) and 14 non-smokers. Full patient characteristics are shown in Table 1.

3.2. EGFR and KRAS Analysis

For the EGFR and KRAS analysis, 53 patients were studied by PCR followed by Sanger sequencing and the remaining 20 were evaluated by MassARRAY (Sequenom) (Figure 1). The EGFR E746-A750 del and EGFR L858R expression was assessed by IHC in 24 patients (Figure 1). Of the 24 patients analyzed by IHC (Table 3), only one (4.16%) harbored an EGFR mutation (E746-A750 del) (Table 5). Abnormal immunolabeling of EGFR was detected in a 70-year old non-smoker male with acinar histological subtype (Figure 2 and Table 3). Cells showing membranous/cytoplasmic staining alone or in association were considered positive and thus scored. None of the patients analyzed by EGFR mutation-specific IHC harbored an L858R mutation. EGFR mutations were also detected by MassARRAY in three (15%) other patients (two males, one smoker and one non-smoker, and one non-smoker female) (Figure 3, Table 5). Four (5.47%) of the 73 patients harbored an EGFR mutation and the E746-A750 del of exon 19 (2235–2249 del) was only detected in three (4.1%) non-smokers (one female and two males) and in one (1.36%) male smoker. A comparison between wild-type and mutant EGFR patients with regard to gender revealed that 1.36% of females and 4.1% of males had an EGFR mutation. In terms of smoking status, 4.1% of non-smokers and only 1.36% of smokers showed an EGFR mutation (Tables 5 and 6). Conversely, KRAS mutations were detected in seven (9.58%) patients of whom six (8.21%) were male (all smokers) and one (1.36%) was female (non-smoker). Three of the KRAS-mutated patients were identified using the MassARRAY (Figure 4A,B) and the remaining four patients were identified using Sanger sequencing (Figure 5). Two of the seven patients showed a G12D mutation, three patients a G13D mutation, one patient a G12A mutation, and one patient a G12V mutation (Tables 5 and 6).

3.3. ALK and p53 Protein Expression Analysis

Of the 73 patients, only one (77-year-old smoker) showed strong granular, homogenous and diffuse ALK cytoplasmic staining (Figure 6 and Table 5). With regard to p53, only nuclear immunostaining was considered. Tumors showing immunoreactivity of >10% of tumor cell nuclei were considered positive (p53 overexpression). Abnormal p53 immunostaining was detected in 41 (56.16%) cases and 21 (28.76%) showed an overexpression of p53 protein (Figure 7).
Figure 2. EGFR immunohistochemistry (IHC) staining in lung adenocarcinoma: (A) Negative immunostaining of EGFR (200×), (B) EGFR cytoplasmic IHC staining in lung adenocarcinoma (LAD). Strong positivity of EGFR in cytoplasm [3+, 100%] (200×).

Figure 3. Spectrum of the Sequenom assay shows EGFR E746-A750 del (exon 19).
Figure 4. Spectrum of the Sequenom assay shows \textit{KRAS} mutations. (A) G12D \textit{KRAS} mutation (exon 2 codon 12), (B) G13D \textit{KRAS} mutation (exon 2 codon 13).

Figure 5. Sequencing by the standard method shows a mutant peak consistent with a G13D, G12A, and G12V mutation.

Figure 6. Anaplastic lymphoma kinase (ALK) protein expression: Tumor harboring positive ALK expression shows strong granular and homogenous cytoplasmic staining, (A) 100×, (B) 250×, (C) 400×.
In the present study we performed a preliminary analysis of the molecular profile of Tunisian patients with lung adenocarcinoma, using different technologies. Our results highlighted the importance of different sequencing approaches (MassARRAY and Sanger sequencing) and the usefulness of IHC in helping to select the best therapeutic strategy. Our investigation revealed that the profile of the different mutations was fairly similar to that of European populations, albeit with a lower prevalence.

The most common EGFR mutations associated with NSCLC (E746-A750 del and L858R), which together accounted for 86–90% of the total number of EGFR mutations (45% for E746-A750 del and 40–45% for L858R) [31–33], were investigated in 24 patients using IHC (Table 1). Abnormal immunolabeling of EGFR was detected in only one (4.16%) case, in contrast to the much higher incidence (44%) recently reported by Mraihi et al. in 50 Tunisian patients [34]. In this context, doing a molecular verification by Sanger sequencing, after performing IHC, would be ideal for the positive case. However, the small size of the biopsy did not allow us to make an additional test. We also used MassARRAY (Sequenom) technology to study the molecular profile of 20 cases, detecting EGFR mutations in three (15%) patients, all of whom harbored the E746-A750 del of exon 19. This incidence was similar to that found in European countries [35]. Furthermore, our results were in agreement with data from a recent Tunisian study by Arfaoui et al. on EGFR mutations determined by Therascreen EGFR RGQ PCR kit (Qiagen) [36] where the authors reported an incidence of 11.5% (3/26 cases) in LAD patients. Conversely, we did not detect any EGFR mutations using Sanger sequencing, which could be attributed to the low sensitivity and need for high-quality tumor samples of this technology [37]. Our results prove that the variability in frequency was primarily related to the sensitivity of the method used to analyze the markers. In general, mutation averages vary from study to study and from country to country, and this variability is probably related to patient selection criteria (clinical and pathological) and to the methods used for mutational analysis. We also observed that EGFR mutations were more frequent in males and non-smokers than in females and smokers (Tables 5 and 6). However, it has been seen that higher EGFR mutation frequencies were observed in female non-smokers of Asian origin [38,39], reaching more than 60% in this population.

Using both Sanger sequencing and Sequenom technology in the second part of our investigation, we observed a similar incidence of KRAS mutations in LAD patients with respect to that previously observed in an Asian population [40]. The frequency was lower than that seen in Caucasian populations (around 30%) [41,42], which may have been due to the smoking habits of the group studied. A recent Chinese study on smokers with LAD reported a 14.0% incidence of KRAS mutations, while that of LAD non-smokers was 3.4% [43]. In our study, KRAS mutations were observed more frequently in male smokers than in female non-smokers (Tables 5 and 6). With regard to ALK expression, we detected positivity in only one (1.36%) of the 73 patients using IHC. Interestingly, our IHC results on ALK differ from those of other studies, independently of the method used. In fact, ALK rearrangements frequencies

![Figure 7. P53 protein expression in lung adenocarcinoma: (A) strong immunostaining (p53 score = 12), (B) low immunostaining (p53 score = 4) (400×).](image)
of 3–5% were observed in unselected patient populations [44–46] and of 33% in highly selected patient populations (EGFR wild type, female, non/light smokers) [47,48]. ALK rearrangement was usually associated with young age, female gender, and a no- or light-smoking history [47,49]. A Tunisian study by Mezni et al. [50] showed a prevalence of ALK rearrangement in six (7.14%) out of 84 patients using IHC, which differs fairly substantially from our findings. Furthermore, Liang et al. [51] suggested that the incidence of ALK rearrangement in the Chinese population may be correlated with smoking status as they observed a lower prevalence in smokers (2.9%) than in non-smokers (7.2%). Smoking and a higher percentage of males in our study may account for the lower incidence of ALK translocations detected. With regard to p53, abnormal immunolabeling was detected in 41 cases (56.16%). There have been some reports of gender differences in p53 mutations [52], and several studies have indicated that TP53 mutations occur more frequently in smokers than in non-smokers [52,53]. In our group, abnormal p53 expression was most frequently observed in males and smokers. However, we did not find a significant correlation between gender or tobacco consumption and p53 mutation.

In summary, our preliminary study highlighted the importance of different sequencing approaches (MassARRAY and Sanger sequencing) and the usefulness of IHC in helping to select the best therapeutic strategy. Furthermore, our investigation revealed that the profile of the different mutations was fairly similar to that of European populations, albeit with a lower prevalence for EGFR, ALK, and KRAS and slightly higher for p53 expression. In addition, our finding encourages the use of the Sequenom as an easy specific and most sensitive approach for the screening of LAD patients, however, this brought us to wonder about the cost especially in low income countries. Further studies with larger Tunisian series are required to obtain more conclusive results.

Table 4. p53 immunostaining score.

| Score | 0 | 1 | 2 | 3 | 4 |
|-------|---|---|---|---|---|
| Staining intensity | No staining | Light yellow | Yellowish brown | Brown | Dark brown |
| Proportion of positive tumor cells | 0% | <10% | 11% to 50% | 51% to 80% | >80% |

Immunostaining score = positive cell proportion score multiplied by staining intensity score

| Degree of immunostaining | 0 | 1–4 | >4 |
|--------------------------|---|-----|----|
|                          | Negative | Low | High |
### Table 5. Clinical pathological data and *EGFR*, *KRAS*, and *ALK* status of patients with genetic alterations.

| Patient No. | Gender | Age, Years | Smoking Data | Specimens | pTNM/Stage Grouping | Histological Subtype | Genetic Alteration | Method |
|-------------|--------|------------|--------------|-----------|---------------------|---------------------|-------------------|--------|
| 1           | M      | 60         | Yes          | Biopsy    | Acinar              | EGFR: EX-19 pE746-A750del | Sequenom |
| 2           | M      | 53         | Yes          | Biopsy    | Acinar              | KRAS G13D           | Sequenom |
| 3           | F      | 61         | No           | Surgical  | pT2bN1M0/II        | Mucinar             | KRAS G12D         | Sequenom |
| 4           | M      | 74         | Yes          | Biopsy    | Lepidic             | KRAS G12D           | Sequenom |
| 5           | F      | 53         | No           | Biopsy    | Solid               | EGFR: EX19 pE746-A750del | Sequenom |
| 6           | M      | 70         | No           | Biopsy    | Acinar              | EGFR: EX19 pE746-A750del | IHC |
| 7           | M      | 71         | No           | Surgical  | pT1N1M0/II         | EGFR: EX19 pE746-A750del | Sequenom |
| 8           | M      | 61         | Yes          | Biopsy    | Lepedid             | KRAS G12A           | Sanger |
| 9           | M      | 66         | Yes          | Biopsy    | -                   | KRAS G13D           | Sanger |
| 10          | M      | 58         | Yes          | Biopsy    | Solid               | KRAS G12V           | Sanger |
| 11          | M      | 62         | Yes          | Biopsy    | Acinar              | KRAS G13D           | Sanger |
| 12          | M      | 77         | Yes          | Biopsy    | -                   | ALK +               | IHC |

IHC, immunohistochemistry.

### Table 6. Genetic alteration in relation to gender and the smoking history of patients with LAD (*n* = 73).

|                  | EGFR+ | KRAS+ | ALK+ | Abnormal p53 Expression |
|------------------|-------|-------|------|-------------------------|
| Gender           |       |       |      |                         |
| Male             | 3     | 6     | 1    | 37                      |
| Female           | 1     | 1     | 0    | 4                       |
| Smoking history  |       |       |      |                         |
| Non-smoker       | 3     | 1     | 0    | 5                       |
| Smoker           | 1     | 6     | 1    | 23                      |

LAD, lung adenocarcinoma.
Author Contributions: D.D. designed the research, carried out all the experiments, and drafted the manuscript; L.C., E.C., and M.C. carried out the molecular analyses and interpreted the results; S.B., I.B., and O.B. performed the IHC analyses; I.Y. collected patient clinical data; R.J. and T.B. were responsible for patient recruitment, pathological data, and IHC analysis/interpretation; P.U., L.A.K., and D.C. critically revised the manuscript for important intellectual content and coordinated the study. All the authors read and approved the manuscript for publication.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments: We are grateful to Kengo Takeuchi from the Japanese Foundation for Cancer Research in Tokyo, Japan, for his help and support. We would like also to thank the staff of the Department of Pathology of CHU Habib Bourghiba, Sfax, Tunisia for their collaboration in the present study. This work was supported by the Ministry of Higher Education and Scientific Research in Tunisia and the University of Sfax.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations
ALK  anaplastic lymphoma kinase
EGFR  epidermal growth factor receptor
FFPE  paraffin-embedded
IHC  immunohistochemistry
KRAS  kirsten rat sarcoma
LAD  lung adenocarcinoma
NSCLC  non-small cell lung cancer
PCR  polymerase chain reaction
TKIs  tyrosine kinase inhibitors

References
1. Pao, W.; Girard, N. New driver mutations in non-small-cell lung cancer. Lancet Oncol. 2011, 12, 175–180. [CrossRef]
2. Author Correction: Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014, 514, 262. [CrossRef]
3. Marchetti, A.; Martella, C.; Felicioni, L.; Barassi, F.; Salvatore, S.; Chella, A.; Campese, P.P.; Iarussi, T.; Muscilli, F.; Mezzetti, A.; et al. EGFR mutations in non-small-cell lung cancer: Analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J. Clin. Oncol. 2005, 23, 857–865. [CrossRef] [PubMed]
4. Tanaka, T.; Matsuoka, M.; Sutani, A.; Gemma, A.; Maemondo, M.; Inoue, A.; Okinaga, S.; Nagashima, M.; Oizumi, S.; Uematsu, K.; et al. Frequency of and variables associated with the EGFR mutation and its subtypes. Int. J. Cancer 2010, 126, 651–655. [CrossRef] [PubMed]
5. Rosell, R.; Moran, T.; Queralt, C.; Porta, R.; Cardenal, F.; Camps, C.; Majem, M.; Lopez-Vivanco, G.; Isla, D.; Provencio, M. Spanish Lung Cancer Group. Screening for epidermal growth factor receptor mutations in lung cancer. N. Engl. J. Med. 2009, 361, 958–967. [CrossRef] [PubMed]
6. Ha, S.Y.; Choi, S.-J.; Ho Cho, J.; Joo Choi, H.; Lee, J.; Jung, K.; Irwin, D.; Liu, X.; Lira, M.E.; Mao, M.; et al. Lung cancer in never-smoker Asian females is driven by oncogenic mutations, most often involving EGFR. Oncotarget 2015, 6, 5465–5474. [CrossRef] [PubMed]
7. Mok, T.S.; Wu, Y.-L.; Thongprasert, S.; Yang, C.-H.; Chu, D.-T.; Saijo, N.; Sunpaweravong, P.; Han, B.; Margono, B.; Ichinose, Y.; et al. Gefitinib or Carboplatin–Paclitaxel in Pulmonary Adenocarcinoma. N. Engl. J. Med. 2009, 361, 947–957. [CrossRef] [PubMed]
8. Yang, J.C.H.; Wu, Y.L.; Schuler, M.; Sebastian, M.; Popat, S.; Yamamoto, N.; Zhou, C.; Hu, C.P.; O’Byrne, K.; Feng, J.; et al. Afitinib versus cisplatin-based chemotherapy for EGFR mutation-positive lung adenocarcinoma (LUX-Lung 3 and LUX-Lung 6): Analysis of overall survival data from two randomised, phase 3 trials. Lancet Oncol. 2015, 16, 141–151. [CrossRef]
9. Rosell, R.; Carcereny, E.; Gervais, R.; Vergnenegre, A.; Massuti, B.; Felip, E.; Palmero, R.; Garcia-Gomez, R.; Pallares, C.; Sanchez, J.M.; et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): A multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012, 13, 239–246. [CrossRef]
10. Soda, M.; Choi, Y.L.; Enomoto, M.; Takada, S.; Yamashita, Y.; Ishikawa, S.; Fujiwara, S.I.; Watanabe, H.; Kurashina, K.; Hatanaka, H.; et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature 2007*, *448*, 561–566. [CrossRef]

11. Inamura, K.; Takeuchi, K.; Togashi, Y.; Nomura, K.; Ninomiya, H.; Okui, M.; Satoh, Y.; Okumura, S.; Nakagawa, K.; Soda, M.; et al. EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. *J. Thorac. Oncol. 2008*, *3*, 13–17. [CrossRef] [PubMed]

12. Zhang, X.; Zhang, S.; Yang, X.; Yang, J.; Zhou, Q.; Yin, L.; An, S.; Lin, J.; Chen, S.; Xie, Z.; et al. Fusion of EML4 and ALK is associated with development of lung adenocarcinomas lacking EGFR and KRAS mutations and is correlated with ALK expression. *Mol. Cancer* 2010, *9*, 188. [CrossRef] [PubMed]

13. Yamaguchi, N.; VanderLaan, P.A.; Folch, E.; Boucher, D.H.; Canepa, H.M.; Kent, M.S.; Gangadharan, S.P.; Majid, A.; Kocher, O.N.; Goldstein, M.A.; et al. Smoking status and self-reported race affect the frequency of clinically relevant oncogenic alterations in non-small-cell lung cancers at a United States-based academic medical practice. *Lung Cancer 2013*, *82*, 31–37. [CrossRef] [PubMed]

14. Shaw, A.T.; Kim, D.-W.; Nakagawa, K.; Seto, T.; Crinó, L.; Ahn, M.-J.; De Pas, T.; Besse, B.; Solomon, B.J.; Blackhall, F.; et al. Crizotinib versus Chemotherapy in Advanced ALK-Positive Lung Cancer. *N. Engl. J. Med. 2013*, *368*, 2385–2394. [CrossRef] [PubMed]

15. Gadgeel, S.M.; Gandhi, L.; Riely, G.J.; Chiappori, A.A.; West, H.L.; Azada, M.C.; Morcos, P.N.; Lee, R.M.; Garcia, L.; Yu, L.; et al. Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002GJ): Results from the dose-finding portion of a phase 1/2 study. *Lancet Oncol. 2014*, *15*, 1119–1128. [CrossRef]

16. Baum, J.; Mick, R.; Zhang, Y.; Watt, C.D.; Vachani, A.; Aggarwal, C.; Evans, T.; Langer, C. Frequency of EGFR and KRAS mutations in patients with non small cell lung cancer by racial background: Do disparities exist? *Lung Cancer 2013*, *81*, 347–353. [CrossRef]

17. Rouquette, I.; Lauwers-Cances, V.; Allera, C.; Brouchet, L.; Milia, J.; Nicoise, Y.; Laurent, J.; Delisle, M.B.; Favre, G.; Didier, A.; et al. Characteristics of lung cancer in women: Importance of hormonal and growth factors. *Lung Cancer 2012*, *76*, 280–285. [CrossRef] [PubMed]

18. Gainor, J.F.; Varghese, A.M.; Ou, S.H.I.; Kabraji, S.; Awad, M.M.; Katayama, R.; Pawlak, A.; Mino-Kenudson, M.; Yeap, B.Y.; Riely, G.J.; et al. ALK rearrangements are mutually exclusive with mutations in EGFR or KRAS: An analysis of 1,683 patients with non small cell lung cancer. *Clin. Cancer Res. 2013*, *19*, 4273–4281. [CrossRef] [PubMed]

19. Won, J.K.; Keam, B.; Koh, J.; Cho, H.J.; Jeon, Y.K.; Kim, T.M.; Lee, S.H.; Lee, D.S.; Kim, D.W.; Chung, D.H. Concomitant ALK translocation and EGFR mutation in lung cancer: A comparison of direct sequencing and sensitive assays and the impact on responsiveness to tyrosine kinase inhibitor. *Ann. Oncol. 2015*, *26*, 348–354. [CrossRef] [PubMed]

20. Rossi, G.; Baldi, L.; Barbieri, F.; Bertolini, F.; Tiseo, M. Concomitant EGFR and KRAS mutations in ALK-rearranged lung cancer. *Ann. Oncol. 2015*, *26*, 1035–1036. [CrossRef]

21. Levine, A.J.; Oren, M. The first 30 years of p53: Growing ever more complex. *Nat. Rev. Cancer 2009*, *9*, 749–758. [CrossRef] [PubMed]

22. Brown, C.J.; Lain, S.; Verma, C.S.; Fersht, A.R.; Lane, D.P. Awakening guardian angels: Drugging the P53 pathway. *Nat. Rev. Cancer 2009*, *9*, 862–873. [CrossRef] [PubMed]

23. Cannon, J.V.; Greaves, R.; Igo, R.; Lane, D.P. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J. 1990*, *9*, 1595–1602. [CrossRef] [PubMed]

24. Hussafvel-Pursiainen, K.; Kannio, A. Cigarette smoking and p53 mutations in lung cancer and bladder cancer. *Environ. Health Perspect.* 1996, *104*, 553–556. [CrossRef] [PubMed]

25. Ettinger, D.S.; Wood, D.E.; Aisner, D.L.; Akerley, W.; Bauman, J.; Chirieac, L.R.; D’Amico, T.A.; Decamp, M.M.; Dilling, T.J.; Dobelbower, M.; et al. Non-small cell lung cancer, version 5.2017: Clinical practice guidelines in oncology. *JNCCN J. Natl. Compr. Cancer Netw. 2017*, *15*, 504–535. [CrossRef]

26. Schaal-Visser, T.B.M.; De Wit, M.; Lam, S.W.; Jiménez, C.R. The cancer secretome, current status and opportunities in the lung, breast and colorectal cancer context. *Biochim. Biophys. Acta Proteins Proteom.* 2013, *1834*, 2242–2258. [CrossRef] [PubMed]

27. Pérez-Caldeo, D.; Romero, A.; Provencio, M.; Torrente, M. Liquid biopsy based biomarkers in non-small cell lung cancer for diagnosis and treatment monitoring. *Transl. Lung Cancer Res.* 2016, *5*, 455–465. [CrossRef]
28. Mlika, M.; Braham, E.; Laabidi, S.; Boussen, H.; El Mezni, F. About the Feasibility of Personalized Medicine in a Low Income Country? *Open J. Soc. Sci.* 2015, 3, 41–45. [CrossRef]

29. Travis, W.D.; Brambilla, E.; Nicholson, A.G.; Yatabe, Y.; Austin, J.H.M.; Beasley, M.B.; Chirieac, L.R.; Dacic, S.; Duhig, E.; Flieder, D.B.; et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances since the 2004 Classification. *J. Thorac. Oncol.* 2015, 10, 1243–1260. [CrossRef]

30. Kato, Y.; Peled, N.; Wynes, M.W.; Yoshida, K.; Pardo, M.; Mascaux, C.; Ohira, T.; Tsuboi, M.; Matsubayashi, J.; Nagao, T.; et al. Novel epidermal growth factor receptor mutation-specific antibodies for non-small cell lung cancer: Immunohistochemistry as a possible screening method for epidermal growth factor receptor mutations. *J. Thorac. Oncol.* 2010, 5, 1551–1558. [CrossRef]

31. Prabhakar, C.N. Epidermal growth factor receptor in non-small cell lung cancer. *Transl. Lung Cancer* 2015, 4, 110–118. [CrossRef]

32. Fan, X.; Liu, B.; Xu, H.; Yu, B.; Shi, S.; Zhang, J.; Wang, X.; Wang, J.; Lu, Z.; Ma, H.; et al. Immunostaining with EGFR mutation-specific antibodies: A reliable screening method for lung adenocarcinomas harboring EGFR mutation in biopsy and resection samples. *Hum. Pathol.* 2013, 44, 1499–1507. [CrossRef][PubMed]

33. Kim, C.H.; Kim, S.H.; Park, S.Y.; Yoo, J.; Kim, S.K.; Kim, H.K. Identification of EGFR mutations by immunohistochemistry with EGFR mutation-specific antibodies in biopsy and resection specimens from pulmonary adenocarcinoma. *Cancer Res. Treat.* 2015, 47, 653–660. [CrossRef][PubMed]

34. Mraihi, Z.; Ben Amar, J.; Bouacha, H.; Rammeh, S.; Hila, L. EGFR mutation status in Tunisian nonsmall-cell lung cancer patients evaluated by mutation-specific immunohistochemistry. *BMC Pulm. Med.* 2018, 18, 132. [CrossRef][PubMed]

35. Moiseyenko, V.M.; Procenko, S.A.; Levchenko, E.V.; Barchuk, A.S.; Moiseyenko, F.V.; Iyevleva, A.G.; Mitiushkina, N.V.; Togo, A.V.; Semionov, I.I.; Ivanstov, A.O.; et al. High efficacy of first-line gefitinib in non-asian patients with EGFR-Mutated Lung adenocarcinoma. *Onkologie* 2010, 33, 231–238. [CrossRef][PubMed]

36. Arfaoui Toumi, A.; Blel, A.; Aloui, R.; Zaibi, H.; Ksentinini, M.; Boudaya, M.S.; Znaidi, N.; Zidi, Y.; Aouina, H.; Rammeh Rommani, S. Assessment of EGFR mutation status in Tunisian patients with pulmonary adenocarcinoma. *Curr. Res. Transl. Med.* 2018, 66, 65–70. [CrossRef][PubMed]

37. Lin, M.T.; Mosier, S.L.; Thiess, M.; Beierl, K.F.; Debeljak, M.; Tseng, L.H.; Chen, G.; Yeegnasubramanian, S.; Ho, H.; Cope, L.; et al. Clinical validation of KRAS, BRAF, and EGFR mutation detection using next-generation sequencing. *Am. J. Clin. Pathol.* 2014, 141, 856–866. [CrossRef][PubMed]

38. Xia, N.; An, J.; Jiang, Q.Q.; Li, M.; Tan, J.; Hu, C.P. Analysis of EGFR, EML4-ALK, KRAS, and c-MET mutations in Chinese lung adenocarcinoma patients. *Exp. Lung Res.* 2013, 39, 328–335. [CrossRef]

39. Gao, B.; Sun, Y.; Zhang, J.; Ren, Y.; Fang, R.; Han, X.; Shen, L.; Liu, X.Y.; Pao, W.; Chen, H.; et al. Spectrum of LKB1, EGFR, and KRAS mutations in Chinese lung adenocarcinomas. *J. Thorac. Oncol.* 2010, 5, 1130–1135. [CrossRef]

40. Takamochi, K.; Oh, S.; Suzuki, K. Differences in EGFR and KRAS mutation spectra in lung adenocarcinoma of never and heavy smokers. *Oncol. Lett.* 2013, 6, 1207–1212. [CrossRef]

41. Arrieta, O.; Cardona, A.F.; Federico Bramuglia, G.; Gallo, A.; Campos-Parra, A.D.; Serrano, S.; Castro, M.; Avilés, A.; Amorin, E.; Kirchuk, R.; et al. Genotyping non-small cell lung cancer (NSCLC) in latin America. *J. Thorac. Oncol.* 2011, 6, 1955–1959. [CrossRef]

42. McQuitty, E.; Zhang, W.; Hendrickson, H.; Tio, E.O.; Jagirdar, J.; Olsen, R.; Cagle, P.T. Lung adenocarcinoma biomarker incidence in hispanic versus non-hispanic white patients. *Arch. Pathol. Lab. Med.* 2014, 38, 390–394. [CrossRef][PubMed]

43. Gou, L.Y.; Niu, F.Y.; Wu, Y.L.; Zhong, W.Z. Differences in driver genes between smoking-related and non-smoking-related lung cancer in the Chinese population. *Cancer* 2015, 17, 3069–3079. [CrossRef][PubMed]

44. Tantraworasin, A.; Lerprasertsutke, N.; Kongkarnia, S.; Euathrongchit, J.; Wannasophya, Y.; Saeteng, S. Retrospective study of ALK rearrangement and clinicopathological implications in completely resected non-small cell lung cancer patients in Northern Thailand: Role of screening with D5F3 antibodies. *Asian Pac. J. Cancer Prev.* 2014, 15, 3057–3063. [CrossRef][PubMed]
45. Minca, E.C.; Lanigan, C.P.; Reynolds, J.P.; Wang, Z.; Ma, P.C.; Cicenia, J.; Almeida, F.A.; Pennell, N.A.; Tubbs, R.R. ALK status testing in non-small-cell lung carcinoma by FISH on ThinPrep slides with cytology material. *J. Thorac. Oncol.* **2014**, *9*, 464–468. [CrossRef] [PubMed]

46. Cabillic, F.; Gros, A.; Dugay, F.; Begueret, H.; Mesturoux, L.; Chiforeanu, D.C.; Dufrenot, L.; Jauffret, V.; Dachary, D.; Corre, R.; et al. Parallel FISH and immunohistochemical studies of ALK status in 3244 non-small-cell lung cancers reveal major discordances. *J. Thorac. Oncol.* **2014**, *9*, 295–306. [CrossRef] [PubMed]

47. Shaw, A.T.; Yeap, B.Y.; Mino-Kenudson, M.; Digumarthy, S.R.; Costa, D.B.; Heist, R.S.; Solomon, B.; Stubbs, H.; Admane, S.; McDermott, U.; et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J. Clin. Oncol.* **2009**, *27*, 4247–4253. [CrossRef] [PubMed]

48. Mattsson, J.S.M.; Brunnström, H.; Jabs, V.; Edlund, K.; Jirström, K.; Mindus, S.; la Fleur, L.; Pontén, F.; Karlsson, M.G.; Karlsson, C.; et al. Inconsistent results in the analysis of ALK rearrangements in non-small cell lung cancer. *BMC Cancer* **2016**, *16*, 603. [CrossRef]

49. Wang, J.; Cai, Y.; Dong, Y.; Nong, J.; Zhou, L.; Liu, G.; Su, D.; Li, X.; Wu, S.; Chen, X.; et al. Clinical characteristics and outcomes of patients with primary lung adenocarcinoma harboring ALK rearrangements detected by FISH, IHC, and RT-PCR. *PLoS ONE* **2014**, *9*, e101551. [CrossRef]

50. Mezni, F.; Mlika, M.; Boussen, H.; Ghedira, H.; Fenniche, S.; Faten, T.; Loriot, M.A. About molecular profile of lung cancer in Tunisian patients. *J. Immunoass. Immunochem.* **2018**, *35*, 256–268. [CrossRef]

51. Fan, L.; Feng, Y.; Wan, H.; Shi, G.; Niu, W. Clinicopathological and demographical characteristics of non-small cell lung cancer patients with ALK rearrangements: A systematic review and meta-analysis. *PLoS ONE* **2014**, *9*, e100866. [CrossRef] [PubMed]

52. Halvorsen, A.R.; Silwal-Pandit, L.; Meza-Zepeda, L.A.; Vodak, D.; Vu, P.; Sagerup, C.; Hovig, E.; Myklebost, O.; Børresen-Dale, A.L.; Brustugun, O.T.; et al. TP53 mutation spectrum in smokers and never smoking lung cancer patients. *Front. Genet.* **2016**, *7*, 85. [CrossRef] [PubMed]

53. Gibbons, D.L.; Byers, L.A.; Kurie, J.M. Smoking, p53 Mutation, and Lung Cancer. *Mol. Cancer Res.* **2014**, *12*, 3–13. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).