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MOLECULAR ANALYSIS AND IMMUNOHISTOCHEMICAL EXPRESSION OF EGFR MUTATIONS IN LUNG ADENOCARCINOMA

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to my three special men

Matteo, Samuele and Federico

I hear babies cry, I watch them grow They'll
learn much more, than I'll never know And I
think to myself, what a wonderful world!

L. Armstrong

SUMMARY

1. INTRODUCTION

1.1 NON SMALL CELL LUNG CANCER

1.1.1 Epidemiology

Lung cancer is the most common cancer and the leading cause of cancer-related death among both men and women in the world. Approximately 1.2 million new cases of lung cancer are diagnosed every year, and 1.1 million patients die of the disease.

Lung cancer accounts for about 13% of all newly diagnosed cancer cases and accounts for about 27% of all cancer deaths. In the United States, it is the second most common cancer, after prostate cancer in men and breast cancer in women. The American Cancer Society projected that 228.190 new cases of lung cancer, including 118.080 men and 110.110 women would be diagnosed in the United States in 2014, with 159.480 deaths (87.260 men and 72.220 women) (Siegel R, 2013).

Lung cancer is more common in men than in women. The age standardizes rate are 72.2 and 51.1 new cases per 100.000 males and females, respectively. In the United States, Northern Europe, and Western Europe, the prevalence of lung cancer has been decreasing in men. In Eastern and Southern European countries, the incidence of lung cancer has been rapidly increasing. Most Western countries have encountered a disturbing trend of increasing prevalence in women and younger patients. Women have a higher incidence of localized disease at presentation and of adenocarcinoma and typically are younger when they present with symptoms (SEER, 2014).

Geographic trends in lung cancer incidence and mortality basically reflect regional differences in smoking behaviour and in wood fires in homes. Developed countries had 723.000 new cases and 599.000 deaths in 2010. In the same year developing nation, including China, reported 833.000 new cases and 778.000 deaths. These data are a reflection of future trends since the rising incidence and mortality of lung cancer will produce huge epidemics in developing countries in the coming decades. In China about half of the male population smokes and 70% of the households use solid fuels for heating and cooking. Tobacco smoking and pollulation in the home from the solid flue usage are the most important global risk factor for chronic obstructive pulmonary disease and lung cancer. These two diseases account for a significant proportion of deaths in developing countries (Yang GH, 2008). The highest incidence occurs in Hungary (51.6 cases per 100.000 population per year), followed by Serbia and the Democratic Republic of Korea. The lowest incidence rates occur in central Africa (approximately 1 case per 100,000 population per year). The highest risk of dying from lung cancer is in Northern America, Australia, New Zealand

and Europe (particularly central and eastern Europe) and South America, while rates in China, Japan and southeast Asia are rising steeply. The lowest rates are observed in Southern Asia and sub-Saharan. Declining lung cancer rates were first observed in the United Kingdom, Finland, Australia, Netherlands, New Zealand, the USA, Singapore, Denmark, Germany, Italy and Sweden. This decline is related to the reduction in smoking especially in the older generations. Lung cancer incidence among women, however, continues to increase in several parts of the globe, although it has begun to plateau in the United States. Notably, despite a very low rate of smoking, Chinese females have a higher incidence of lung cancer than European females (SEER, 2014).

Cigarette smoking is the most important risk factor for lung cancer. However, global statistics estimate that 15% of lung cancers in men and 53% in women are not attributable to smoking, overall accounting for 25% of all lung cancer cases worldwide. Lung cancer in never smokers would rank as the seventh most common cause of cancer death worldwide. Epidemiological studies of lung cancer in never smokers have identified significant gender and geographic variations. A consistent global observation is that lung cancer in never smokers occurs more frequently in women than men. In addition, it has been observed that the proportion of female lung cancer cases in never smokers varies considerably from region to region. For example, the proportion of females with lung cancer who are never smokers is as high as 83% in South Asia (Badar F, 2006), whereas approximately 15% of females with lung cancer in the United States are never smokers (Cerfolio RJ, 2006; Wakelee HA, 2007). Among men, the proportion of never smokers is lower with less regional variation (Brennan P, 2006; Toh C, 2006; Wakai K, 2006).

Lung cancer mainly occurs in older people. About 2 out of 3 people diagnosed with lung cancer are 65 or older; no more than 10% of lung cancers are diagnosed in individuals under 50 years of age and in less than 2% of people younger than 45 years of age. The median age at the time of diagnosis in the USA is about 70 years. (SEER, 2014). There is a greater representation of African-Americas, Asians and pacific islanders in the < 40 age group. Most tumors are adenocarcinomas and while many patients have significant smoking histories, up to 34% are never smokers (Subramanian J, 2010). This percentage of no smokers does not appear to differ from that in the older populations. Nevertheless, environmental, hormonal and genetic factors are probably codeterminants.

1.1.2 Risk and protective factors
Cigarette smoking is the most important risk factor for lung cancer, and it is considered to be responsible for 85% of lung cancer in men and 47% of lung cancer in women. The risk of lung

cancer in cigarette smokers depends on duration of smoking, number of cigarettes smoked, type of cigarette smoked and inhaling pattern. The relative risk for lung cancer between smokers with respect to non smokers is 15 and 30 times.

Although the risk of lung cancer in former smokers remains higher than in individuals who have never smoked, the risk for former smokers decreased over time. The benefit of cessation becomes apparent approximately 5 years after quitting and increases as the length of abstinence lengthens. Passive smoking carries a risk of developing lung cancer. It has been estimated a relative risk between 1.14 to 5.20 in people who had never smoked but who lived with a smoker (Zhong L, 2000).

Tobacco smoking increases the risk of all major histological types of lung cancer, but appears to be strongest for squamous cell carcinoma, followed by small cell carcinoma and adenocarcinoma. The association between adenocarcinoma and smoking has become stronger over time, and adenocarcinoma has become the most common type in many Western countries.

Cigar, cigarillo and marijuana smokers are at greater risk of developing lung cancer than non smokers, but lung cancer risk is less strongly associated with cigar than with cigarette smoking (Shaper AG, 2003; Henley SJ, 2004).

Radon gas, a radioactive gas that is produced by the decay of radium 226, is the second leading cause of lung cancer. The decay of this isotope leads to the production of substances that emit alpha-particles, which may cause cell damage and therefore increase the potential for malignant transformation (Krewsky D, 2005; Darby S, 2005).

Asbestos, a mineral compound that breaks into small airborne shards, is a known carcinogen that increases the risk for lung cancer in people exposed to the airborne fibers, especially those who smoke. An estimated 3% to 4% of lung cancers are caused by asbestos exposure

Lung cancer could be one of the long-term adverse effects of cumulated exposure to ambient air pollution, such as emissions rich in various polycyclic aromatic hydrocarbon compounds, likely through oxidative stress, inflammation, induction of a procoagulatory state, and dysfunction of the autonomic nervous system. The proportion of lung cancers attributable to urban air pollution in Europe is estimated to be 11% (Boffetta P, 2006).

Many work settings could have exposed workers to carcinogens, leading to an increased risk of lung and other cancers. Crystalline silica and chrysotile asbestos are well known human

carcinogens; as expected, workers exposed to silica dust and asbestos fiber are at a higher risk of developing lung cancer. Uranium miners and nuclear plant workers are also known to have an increased cancer risk because of exposure to radioactive particulate mass (Boffetta P; 2006).

Other possible risk factors include recurring lung inflammation, lung scarring secondary to tuberculosis, family history, and exposure to other carcinogens, such as bis(chloromethyl) ether, polycyclic aromatic hydrocarbons, chromium, nickel, and organic arsenic compounds. Some substances are recognized as lung carcinogens (asbestos, chrome, arsenic, beryllium, vinyl cloride, etc) and they can potentiate their effect in presence of tobacco smoke.

Although it has been estimated that 15-25% of human cancer may have a viral aetiology, two viruses, the human papilloma virus (HPV) and Jaasiekte sheep retrovirus (JSRV), have been speculated to have a role in the pathogenesis of lung cancer (Fei Y, 2006; Leroux C, 2007).

Fruits and vegetables that are a rich source of antioxidant vitamins and other micronutrients, particularly carotenoids, are thought to benefit health by decreasing the risk of lung cancer. In contrast, cured meat (eg, sausage, pressed duck, and cured pork), deep fried cooking, and chilli have been associated with an increased lung cancer risk (Ruano-Ravina A, 2006).

Available data suggest that physically active individuals have a lower risk of lung cancer: moderate to high levels of leisure time physical activity were associated with a 13% to 30% reduction in lung cancer risk. Overall, physical activity could help to reduce lung cancer risk and mortality among heavy smokers (Tardon A, 2005).

Although lung cancer is commonly considered a disease caused by environmental exposures, genetic factors are also thought to have a role. The observation that only 10-20% of smokers develop lung cancer suggests that individuals might differ in their susceptibility to environmental risk factors.
Rare Mendelien cancer syndromes of some tumor suppressor genes are associated with a marked increased lung cancer risk. In families with the Li-Fraumeni syndrome or with RB1 mutation, smokers are at much higher risk of lung cancer than non smokers carrying the same mutation (Hwang SJ, 2003; Fletcher O, 2004).
Genetic epidemiological studies haven suggested that individuals with cytochrome P450 1A1 (CYP1A) polymorphisms (I462V) and/or a homozygous deletion of glutathione S-transferase

(GSTM1) are at increased risk of lung cancer (Cote ML, 2007). Pooled analyses of case control studies of CYP1A1 and GSTM1 polymorphisms have found that the CYP1A1-I462V polymorphism is associated with a twofold to threefold increased risk of lung cancer in Caucasian (but not in Asian) never smokers with no significant effect of the GSTM1 null genotype.

Differences in DNA repair capacity might also contribute to susceptibility to lung cancer, with the underlying hypothesis that individuals with lower capacity to repair DNA have a higher risk of lung cancer from DNA-damaging carcinogens. Polymorphisms in DNA repair genes involved in base excision repair (XRCC1 and OGG1), nucleotide excision repair (ERCC1, ERCC2 and XPA), DNA double-strand break repair (XRCC3) and mismatch repair pathways (MLH1 and MSH2) have been studied in association with lung cancer risk. The role of polymorphisms in DNA repair genes could be dependent on smoking status.

1.1.3 Signs and symptoms

Sign and symptoms associated with pulmonary carcinomas depend on tumor location and extent, as well as tumor biology (Patel AL, 1993). Patients with lung cancer present with progressive shortness of breath, cough, chest pain/oppression, hoarseness or loss of voice, haemoptysis (mostly with squamous cell carcinoma). Pneumonia (often recidivant) is the presenting feature in many patients. Relative to other forms of non small cell lung cancer, adenocarcinoma is more often asymptomatic.

Symptoms related to disseminated disease include weight loss, abdominal pain due to involvement of the liver, adrenals and pancreas, and pain due to bone (marrow) metastases. Paraneoplastic symptoms are common in lung cancer.

1.1.4 Classification

Lung carcinomas, according to the current 2004 World Health Organization classification (Travis WD, 2004), are subdivided into small cell lung carcinoma and non small cell lung carcinomas (NSCLC), the letter representing more than 85% of lung cancers.

NSCLC encompass three main histological group: adenocarcinoma, squamous cell carcinoma and large cell carcinoma, and minor entities.

Adenocarcinoma of the lung

Adenocarcinoma (ADC) is the commonest histological subtype of lung cancer both in males and females, smokers and no-smokers, in most countries of the world and accounts for almost half all lung cancer. ADC is also the most common type of lung cancer seen in non-smokers. Sixty four to

70% of never smokers who develop lung carcinoma have ADC while 30 to 42% of smokers have this type of cancer (Wakelee HA, 2007). Almost 53% of lung cancers in women and 15% in men are not tobacco-related. Observational pathology studies report that from 58% to 72% of small ADC are diagnosed in women and more than half of these women are non smokers (Travis WD, 2011).

Adenocarcinoma is a malignant epithelial tumours with glandular differentiation and/or mucin production and express biomarkers that are consistent with an origin in the distal lung, including thyroid transcription factor 1, citokeratin 7, napsin A and surfactant antibodies A.

Major advances in epidemiological, radiological, histological, immunohistochemical and molecular research suggest that ADCs encompass a wide spectrum of different entities.

The WHO adenocarcinoma subclassification was expanded from three entities in 1967 to five major subtypes and five variants in 2004: pure forms of BAC, acinar, papillary and solid type and lastly the mixed subtype which is a heterogeneous tumour group that have variable combinations of 2 or more histologic subtypes patterns. The term BAC was become unwieldy and confusing for pathologists and clinicians. Some use it as a diagnosis others as a descriptive term. The mixed subtype was also problematic since at least 80% of lung adenocarcinoma belong in this category. Thus the classification could not separate possibly distinct subgroups and did not allow for morphological-radiological-molecular studies on particular architectural patterns. These problems interfered with target therapy choices for patients with advanced carcinoma.

Because of remarkable advances over the last years in understanding of lung adenocarcinoma, particularly in area of medical oncology, molecular biology, and radiology, there was a need for a revised classification, based not on pathology alone, but rather on an integrated multidisciplinary platform. The goal was not only longer to solely provide the most accurate diagnosis but also to manage the tissue in a way that immunohistochemical and/or molecular studies could be performed to obtain predictive and prognostic data that will lead to improvement in patient outcomes.

For these reasons in 2011 the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS) published a new International Multidisciplinary lung adenocarcinoma classification (Travis WD, 2011).

Primary aims of the new classification included provision of consistent terms and diagnostic criteria for ADC subtypes, particularly for BAC and mixed subtype ADC, and incorporation of significant practice changing advances in the fields of pathology, molecular biology, oncology, radiology and surgery in to classification that is still principally based on histopathologic examination (Table 1.).

Some of the most important changes included making the terms BAC and mixed subtype adenocarcinoma obsolete. For resection specimens, new concepts was introduced such as adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA) for small solitary adenocarcinomas with either pure lepidic growth (AIS) or predominant lepidic growth with <5 mm invasion (MIA) to define patients who, if they undergo complete resection, will have 100% or near 100% disease specific survival, respectively. AIS and MIA are usually non mucinous but rarely may be mucinous.

Invasive adenocarcinomas were classified by predominant pattern after using comprehensive histologic subtyping with lepidic (formerly most mixed subtype tumors with nonmucinous BAC), acinar, papillary, and solid patterns; micropapillary was added as a new histologic subtype. Variants include invasive mucinous adenocarcinoma (formerly mucinous BAC), colloid, fetal, and enteric adenocarcinoma.

This classification also provided guidance for small biopsies and cytology specimens, as approximately 70% of lung cancers are diagnosed in such samples. Non-small cell lung carcinomas (NSCLCs), in patients with advanced-stage disease, should be classified into more specific types such as adenocarcinoma or squamous cell carcinoma whenever possible and the use of the term NSCLC not otherwise specified should be minimized for several reasons: adenocarcinoma or NSCLC not otherwise specified should be tested for epidermal growth factor receptor (*EGFR*) mutations as the presence of these mutations is predictive of responsiveness to EGFR tyrosine kinase inhibitors; adenocarcinoma histology is a strong predictor for improved outcome with pemetrexed therapy compared with squamous cell carcinoma, and potential life-threatening hemorrhage may occur in patients with squamous cell carcinoma who receive bevacizumab. If the tumor cannot be classified based on light microscopy alone, special studies such as immunohistochemistry and/or mucin stains should be applied to classify the tumor further.

Table 1. IASLC/ATS/ERS Classification of LungAdenocarcinoma in resection specimens

Preinvasive lesions
    Atypical adenomatous hyperplasia
    Adenocarcinoma in situ (≤3 cm formerly BAC)
        Nonmucinous
        Mucinous
        Mixed mucinous/nonmucinous
Minimally invasive adenocarcinoma (≤3 cm lepidic predominant tumor
        with ≤5 mm invasion)
    Nonmucinous
    Mucinous
    Mixed mucinous/nonmucinous
Invasive adenocarcinoma
    Lepidic predominant (formerly nonmucinous BAC pattern, with >5 mm
        invasion)
    Acinar predominant
    Papillary predominant
    Micropapillary predominant
    Solid predominant with mucin production
Variants of invasive adenocarcinoma
    Invasive mucinous adenocarcinoma (formerly mucinous BAC)
    Colloid
    Fetal (low and high grade)
    Enteric

BAC, bronchioloalveolar carcinoma; IASLC, International Association for the Study of Lung Cancer; ATS, American Thoracic Society; ERS, European Respiratory Society.

Given their morphological heterogeneity, it is highly unlikely that pulmonary ADC arise from a single cell type. Expression profile studies suggest two separate histogenetic origins of lung ADC. Clara cells, non ciliated bronchiolar cells and type II pneumocyte in the peripheral lung represent one group of progenitor cells. Basal cells and mucous cells from the central conducting airways are probably the other tumor progenitor cells. These terminal respiratory unit (TRU) and non-TRU cluster respectively appear to represent the two major subtypes of ADC. (Weir BA, 2007). Interestingly the TRU subclass contains a large number of female non or light smokers with high frequency of EGFR mutations.

1.1.5 Genetic mutations

Several molecular and genetic studies have revealed that multiple genetic and epigenetic changes are found in clinically evident lung cancers, involving known and putative tumor suppressor genes as well as several dominant oncogenes.

Recent advances in next-generation sequencing and other high-throughput genomic profiling platforms have allowed researchers to examine the breadth of genetic mutations within lung tumours. Following the identification of KRAS and BRAF mutations (Davies H, 2002; Santos E; 1999), epidermal growth factor receptor (EGFR) mutations were discovered in patients with lung adenocarcinoma and were associated with response to EGFR inhibitors (Lynch TJ, 2004; Pao W, 2004; Shepherd FA, 2005). Further recurrent mutations and amplifications in many potentially targetable oncogenes have since been identified in lung adenocarcinoma, including HER2 (also known as ERBB2), MET, fibroblast growth factor receptor 1 (FGFR1) and FGFR2, as well as fusion oncogenes involving anaplastic lymphoma kinase (ALK), the ROS1 receptor tyrosine kinase, neuregulin 1 (NRG1), neurotrophic tyrosine kinase receptor type 1 (NTRK1) and RET (Zhao C, 2014). These oncogenic changes, many of which predict sensitivity to clinical inhibitors, jointly account for most cases of lung adenocarcinoma (Ding L, 2008; Imielinski M, 2012). For lung squamous cell carcinoma (SCC), the number of tumours for which whole-exome sequencing is available is lower than for ADC but, so far, potentially targetable mutations in adenocarcinoma do not seem to be prevalent in this histological subtype. Instead, genes such as discoidin domain containing receptor 2 (DDR2), FGFR1, FGFR2, FGFR3 and genes in the PI3K pathway seem to be more commonly mutated in lung SCC. Many of these mutations (with the exception of those in the PI3K pathway) have been validated by preclinical studies as driver mutations.

Several tumor suppressor genes, such as TP53, RB, p16INK4a, DUTT1, FHIT, FUS-1 and BAP-1 gene show frequent abnormalities in lung cancer (Wistuba JC, 2006).

Next-generation sequencing studies have also revealed the molecular taxonomy of lung cancer and have shown a dazzling complexity of somatic alterations in NSCLC that extends far beyond protein kinases to include epigenome modifiers, transcription factors, splicing factors and genes involved in cellular immunity (Kandoth C, 2013).

1.1.6 Staging and TNM classification

Staging is one of the most important components in the management of lung cancer. Accurate staging is important because it allows the clinician to predict prognosis and assign appropriate therapy and also provides a system that allows clinicians and researchers to stratify patients into reasonably homogenous groups so that treatment outcomes can be appropriately compared.

Pathologic stage refers to the best prediction of stage following pathologic analysis of the patient's tumor, lymph nodes, and/or metastases and is usually applied following surgical resection or exploration.

The last updated staging system is the 7th edition of the American Joint Commission on Cancer (AJCC) and the International Union Against Cancer (UICC) Staging Manual (Goldstraw P, 2010).

1.1.7 Prognosis

Lung cancer is the leading cause of cancer-related death among both men and women in the world. Despite advances in treatment, the 5-year survival rate for NSCLC across all stages is only 15% (Jemal A, 2008). The most important clinical prognostic factors are disease stage, performance status, age and sex. Among the histological parameters, those that correlate with a low survival are high histological grade, vascular invasion, the high mitotic index and necrosis.

The stage of the tumor still remains one of the most important prognostic factor. The IASLT staging project (Goldstrow P, 2010) reports overall 5-year survival of 73% for stage IA, 58% for stage IB, 46% for stage IIA, 36% for stage IIB, 24% for stage IIIA and 9% for stage IIIB following surgery and 13% for stage IV non small cell lung cancer patients. The aggressive nature of non small cell carcinoma is starkly reflected in the fact that no more than 30% of patients who are stages I to IIIA are amenable to potentially curative surgical resection at initial diagnosis. Twenty-seven percent of patients with stage IA cancer and 42% of those with stage IB non small cell carcinoma relapse and die of their disease.

The different outcome of patients within the same stage of NSCLC suggests to investigate further the biological pathway of these tumors to predict recurrences and to optimise the therapeutic interventions. Many biological and molecular markers have been found to have prognostic value for survival in NSCLC and some of them are now being used to stratify patients for treatment.

1.1.8 Therapies

The treatment options for non-small cell lung cancer (NSCLC) are based mainly on histological type (non-small cell versus small cell carcinoma and adenocarcinomaa versus squamous cell carcinoma), tumor stage, molecular characteristics and the performance status.

Patients with stage I, II, or III non-small cell lung cancer (NSCLC) are generally treated with curative intent using surgery, chemotherapy, radiation therapy (RT), or a combined modality approach.

For early stage NSCLC, surgery is the most important curative modality. However, only approximately 20-30% of patients are diagnosed at resectable stage (stage I-II). For advanced NSCLC patients with good performance status, platinum-based chemotherapy represents the standard treatment and partial responses can be achieved in 30-40% of the cases while complete responses are very rare (Azzoli CG, 2011). Combinations with cisplatin or carboplatin, and third generation cytotoxic drugs, such as gemcitabine, paclitaxel, docetaxel, etoposide or vinorelbine, are used. Moreover, both chemo- and radiotherapy can be used in neo-adjuvant setting to shrink the tumor before surgery or as adjuvant therapy to improve outcome after resection. The use of both neo-adjuvant and adjuvant chemotherapy has been shown to improve patient survival (Kim

AW, 2011; Douillard JY, 2006). Despite advances in these combined treatment modalities for lung cancer, prognosis remains poor and severe side effects are often observed. Therefore, more effective and less toxic treatments are needed and, as a result, a variety of molecular targeted therapies have been recently introduced for the treatment of advanced NSCLC.

Targeted therapies

The novel targeted therapies are based on advances in our understanding of key cellular networks and genetic nodal points around which tumors could arise and progress (Herbst RS, 2008). Genome characterization efforts have highlighted the importance of "driver" somatic alterations that activate crucial oncoproteins originating tumor with a pivotal dependency. Single-agent therapeutic regimens especially designed to intercept deregulated dominant oncogenes have proven to be effective treatment in these "oncogene addicted" tumors.

A number of molecular aberrations have been identified in NSCLC including EGFR mutations/amplifications, EML4-ALK translocation fusions, KRAS mutations, PIK3CA mutations, IGF-1R overexpression, and MET amplification/overexpression. Conflicting results have demonstrated marginal benefit with targeted agents in unselected populations of patients with advanced NSCLC. However, many targeted agents have been approved in different line setting for the treatment of specific subgroups of patients. In particular, the epidermal growth factor receptor (EGFR) has been successfully targeted in this disease either by monoclonal antibodies (mAbs) or small molecules inhibiting the tyrosine kinase domain (gefitinib and erlotinib). Cetuximab is a mAb which blocks the extracellular domain of EGFR, thereby competing with the ligands, resulting in the inhibition of the receptor. This mAb, which is approved for the treatment of advanced colorectal cancer, has also been approved as first-line treatment combined with platinum-based chemotherapy in EGFR-positive NSCLC patients with good performance status (Pirker R, 2009).

Gefitinib has been approved by U.S. Food and Drug Administration (FDA) and EMEA as upfront therapy replacing chemotherapy in late-stage NSCLC patients harboring activating-EGFR mutations (Ku GY, 2011). Furthermore, the manageable toxicity, along with its efficacy, make erlotinib an important option as maintenance therapy, and both erlotinib and gefitinib are also the only drugs of proven efficacy in the third-line setting for patients with NSCLC previously treated with chemotherapy. Another example of targeted therapy for the treatment of advanced NSCLC is the antiangiogenic agent bevacizumab, in combination with carboplatin-paclitaxel or any platinum-based chemotherapy, which has been recently approved as first-line treatment for patients bearing tumors with non-squamous histology. More recently the anaplastic lymphoma kinase (ALK) inhibitor crizotinib has also been approved by FDA for the treatment of locally

advanced or metastatic NSCLCs that express the abnormal ALK gene (Gandhi L, 2012). Aberrations in other key molecules and signaling pathways, such as RAS/RAF/MEK, PI3K/Akt/mTOR, or c-MET, have been identified as crucial targets, especially in resistant patients. Novel drugs aimed to interact with these abnormal molecules are actively being investigated in the clinic, including the ROS1, FGFR1, RET, TRK, BRAF inhibitor, and many others (Morgensztern D, 2015).

1.2 EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptor (EGFR) is a 486 aminoacid receptor protein of 170 kDa with a single transmembrane sequence between 4 extracellular and 3 intracellular domains. Ligand-binding activity is in the extracellular domain 3. EGFR ligands include EGF, transforming growth factor-alpha, and amphiregulin (Khazaie K, 1993). The binding of EGFR ligands induces dimerization allowing for receptor phosphorylation; such dimers can be homodimers or heterodimers with other HER family receptors (Ullrich A, 1990). Several members that belong to the ErbB family have been described: HER1 (EGFR/erbB1), HER2 (neu, erbB2), HER3 (erbB3), and HER4 (erbB4) (Carpenter G, 1987). These different receptors display a specific distribution and molecular alteration pattern depending on the tumor entity. The intracellular component of the EGFR protein includes a juxtamembrane domain, a region housing the TK activity, and a C-terminal domain. The kinase activity is dependent on a lysine residue at amino acid 721, but key components affecting ATP affinity are found within that domain. Alterations in the juxtamembrane domain and C-terminal domains can influence ligand binding. More importantly, numerous tyrosine residues are present in the C-terminal domain, and phosphorylation in these sites influence protein–protein interactions that are essential for signal transduction via pathways such as those mediated by PI3K and rasGAP/KRAS (Ullrich A, 1990).

Although each domain has specific function, truncation of exons 2-7 can cause a cell lineage specific activation, which can also be associated with amplification. This alteration was described in 1985 in gliomas, and for many years this was the best demonstration of the oncogenic potential of constitutive EGFR activation (Libermann TA, 1985). This alteration affects receptor recycling and results in constitutive activity that is independent of ligand binding (Huang HS,1997).

Dimerization of EGFR leads to TK activity and receptor phosphorylation. This phosphorylation induces interaction with GRB2, then SOS that leads to RAS activation. What follows is a cascade of kinase activations including BRAF and MEK1, ultimately resulting in ERK1/2 phosphorylation and activation (Liebmann C, 2001). This signaling pathway has effects on cellular proliferation.

In addition, the PI3K pathway is activated, causing an increase of its downstream effector protein kinase-B, also referred as AKT (Jorissen RN, 2003). Dephosphorylation of AKT is regulated by PTEN, a pivotal phosphatase with tumorsuppressive properties. PTEN is known to be mutated (for example, homozygous deletions) or epigenetically silenced in several malignancies, including glioblastoma and uterine endometrioid adenocarcinoma. PTEN possesses also the capability to mediate activation status of EGFR by influencing EGFR phosphorylation status (Sos ML, 2009). AKT itself regulates apoptotic signaling by phosphorylating and driving the expression of certain anti-apoptotic molecules, such as the inhibitor of apoptosis protein surviving (Guo Y, 2013) (Figure 1.). An additional important downstream target is the mTOR pathway that affects protein translation, and is implicated in cell cycle progression, apoptosis, and metastasis. Being often constitutively active in several highly treatment refractory cancers, the mTOR pathway has also emerged as a target in cancer therapy. STAT signaling is also affected by EGFR signaling. For instance, STAT3 is phosphorylated and activated by activating EGFR mutations in lung adenocarcinomas (Takata S, 2012).

1.2.1 Primary EGFR mutations and TK inhibitor therapy

Activating mutations in oncogenes have been described as an 'Achilles heel' in cancer, coining a term of 'oncogene addiction' (Weinstein IB, 2006). Dependency of a tumor on a specific oncogene renders these malignancies potentially sensitive to inhibitors that preferentially target the altered oncogene and thereby abrogates the tumor-promoting properties of it. In fact, sudden cessation of signaling can induce cell death.

Targeting EGFR in lung cancer began before knowledge of the existence of specific activating mutations. Gefitinib, when used in unselected populations, still resulted in a response rate of 10–20% (Fukuoka M, 2003; Kris MG, 2003). Erlotinib administration was associated with an improvement in overall survival, but this important finding was overshadowed by more dramatic results in certain subgroups adenocarcinoma histology, female gender, nonsmoking status, and Asian ethnicity (Sheperd FA, 2005). This finding of patients with lung adenocarcinomas with response to gefitinib and erlotinib (EGFR TK inhibitors or EGFR TKI) led to molecular investigation of the EGFR pathway in these tumors.

In studies published in 2004, several mutations that render lung adenocarcinomas sensitive to gefitinib were identified (Lynch TJ, 2004; Pao W, 2004). The fraction of patients who were sensitive to gefitinib were subjected to mutation analysis of the EGFR gene and eight of nine patients showed a mutation in the ATP-dependent TK domain ('catalytic kinase domain') in the EGFR gene (Lynch TJ, 2004). Most of these mutations were point mutations or in-frame deletions, which were present in a heterozygous pattern. Specifically, they found three point mutations

(G719C, L858R, and L861Q) and three main in-frame deletions (delE746-A750, delL747-T751inS, and delL747- P753inS). This group also carried out a functional analysis of two of the identified mutations (L858R and delL747-P753insS) and ectopically overexpressed these two mutations and wild-type EGFR in COS-7 cells. In response to the EGFR ligand EGF, Cos-7 cells harboring the mutations had a higher phosphorylation activity of EGFR (assessed by immunoblotting for the EGFR tyrosine-residue Y1068) and were more susceptible to gefitinib as compared with wildtype EGFR-expressing Cos-7 cells, corroborating the notion that tumors with these indicated mutations may be more responsive to EGFR TKI treatment.

The distribution of these mutations around the 'catalytic kinase domain' is distinct in lung adenocarcinoma and contrasts the mutations in glioblastomas that are located in the extracellular portion of EGFR.

A larger study involving 617 NSCLC tumor samples identified mutations in the EGFR gene in 21% of the cases and confirmed the initial findings. These mutations were predominantly observed in adenocarcinomas, more common in non smokers and in patients from east Asia. (Shigematsu H, 2005).

Separate studies addressed the important issue of whether EGFR TK domain mutations are an early event in the development of lung adenocarcinoma and may represent a target for 'chemoprevention strategies' (Tang X, 2005).

From the current body of available literature, three defined regions (exons 18-21) in the EGFR gene are commonly mutated and predict sensitivity to EGFR TKI (Figure 2.).

The most common of these mutations in the EGFR gene involve the point mutation, L858R, in exon 21 and an exon 19 deletion, del746-750. These comprise up to 86% of EGFR mutations in NSCLCs overall. Concerning the sensitivity of these above-mentioned mutations, it has been suggested that in-frame deletions of EGFR predict higher sensitivity to TKI compared with point mutations in exons 18 and 21 (Eberhard DA, 2005; Sakurada A, 2006).

Additional mutations in the EGFR gene have been identified, in exons 20 and 18. Given the paucity of these rarer mutations, their impact on predicting sensitivity or resistance to EGFR TKI remains to be fully elucidated in larger patient populations (Eberhard DA, 2005).

However, based on current knowledge, some observations have been made regarding rarer mutations and EGFR TKI therapy. The presence of L861Q mutations may be associated with stable disease, and exon 20 insertions are reported as a potential cause of TKI resistance (Yang CH, 2008; Girard N, 2010).

Although T790M mutations are found in up to 63% of EGFR TKI-treated tumors as a form of acquired resistance primary resistance due to T790M mutations is thought to be less frequently encountered, with conflicting results using EGFR (Siegelin M, 2014).

In rare instances, the T790M mutation is a germline mutation. In a very small subset of patients with NSCLCs this alteration causes primary resistance to EGFR TKIs (Girard N, 2010). To further explore the mechanism of how EGFR inhibitors elicit cell death in lung adenocarcinomas harboring EGFR sensitizing mutations, several groups hypothesized that gefitinib may elicit apoptotic cell death, depending on the Bcl-2 family of proteins (Gong Y, 2007). In addition to apoptosis, other forms of cell death have been described in the context of EGFR inhibition such as autophagy.

1.2.2 Resistance pathways

Acquired EGFR Mutations in Lung Adenocarcinom as after EGFR Receptor Inhibitor Treatment

Despite the remarkable response of EGFR mutant lung adenocarcinomas to TKIs, essentially all these tumors recur and eventually develop secondary evolved resistance. These resistant tumors have commonly acquired TKI treatment related EGFR mutations. One of these mutations is located in exon 20 of the EGFR gene and replaces methionine for a threonine (T790M) (Kobayashi S, 2005) resulting in increased ATP affinity. This mutation was identified in a patient, who initially responded well to gefitinib treatment (with complete remission) and had an exon 19 mutation in EGFR (delL747–S752). After 2 years the tumor recurred, retaining the initially identified exon 19 deletion, but had acquired additionally a second point mutation of the EGFR TK domain in exon 20 (T790M).

EGFR T790M mutations are associated with poorer response and shorter PFS (Rosell R, 2011).

Aside from mutational pathways, activation of survival pathways also contributes to EGFR TKI resistance. Specifically, MET signaling and the amplification of the MET receptor have been shown to hamper the effectiveness of EGFR inhibitors in patients with lung adenocarcinomas (Cheng TL, 2005; Engelman JA,2007; Yano S, 2008; Turke AB, 2010).

As a further mechanism of acquired resistance, HER2 amplification was recently described as a contributing factor in 13% of 155 cases (Yu AH, 2013).

1.2.3 Non responders to EGFR TK Inhibitor

Certain mutations of the EGFR downstream signaling confer resistance to EGFR inhibitor treatments. The most prominent member out of this group is KRAS gene. KRAS and EGFR mutations generally occur in a mutually exclusive pattern, although some cases have been identified in which both EGFR and KRAS were mutated in the same tumor (Takeda M, 2010). Despite harboring sensitizing EGFR mutation, patient having mutations in both genes were nevertheless resistant to EGFR inhibitor treatment. Therefore, KRAS mutation predicts non

response to EGFR TKI either as a result of the mutually exclusive occurrence or as the fact that pathway activation becomes at least partly independent of EGFR activity.

Several others alteration are also seen in EGFR wild-type tumors and predicts non response to EGFR TKI therapy (Siegelin MD, 2014): loss of PTEN, mutations in BRAF, PIK3CA, MEK1 and ERBB2, and translocations involving anaplastic lymphoma kinase (ALK), ROS1, and RET.
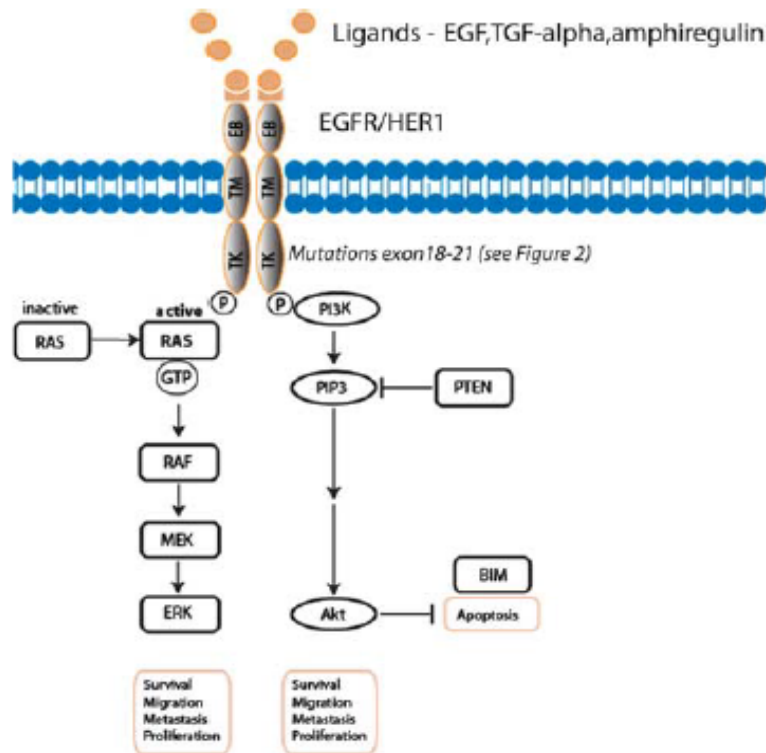

Figure 1. Epidermal growth factor receptor (EGFR) pathway.

Figure 2. Epidermal growth factor receptor (EGFR) mutations and their frequency based on COSMIC annotations.

2. AIM OF THE STUDY

According to the National Comprehensive Cancer Network (NCCN) guidelines, EGFR-TKIs have been approved as first-line therapeutic agents for patients with advanced, recurrent, or metastatic NSCLC with mutated EGFR (Kim HJ, 2012; Kobayashi K, 2013; Wang S, 2013).In these patients, since surgical resection is not recommended, small biopsy or cytological specimens are the only materials available for EGFR mutation testing (da Cunha Santos G, 2011; Moskalev EA, 2013; Sun PL, 2013). These specimens often contain a small amount of tumor cells, which can be a serious obstacle to the reliable detection of EGFR mutations using DNA sequencing methods.

Yu et al. reported that mutation-specific antibodies against E746-A750 del and L858R could be used to detect 2 major mutations of EGFR by using immunohistochemistry (IHC) on formalin-fixed paraffin-embedded tissues (Yu J, 2006). IHC is an easy, rapid, well-established, and routinely performed method that can be used irrespective of the tumor volume and DNA degradation in laboratories (Kawahara A, 2011; Xiong Y, 2013). The accuracy of mutation-specific antibodies has been evaluated in several studies. While the results for their sensitivity are inconsistent, there is high consistency with regard to their specificities (Brevet M, 2010; Kato Y, 2010; Kitamura A, 2010; Simonetti S, 2010; Kawahara A, 2011; Wu SG, 2011; Xiong Y, 2013).

Novel antibodies, SP111 for E746-A750 del and SP125 for L858R, have been recently developed.

In this study we investigated the accuracy of two new mutation-specific monoclonal antibodies, SP111 and SP125, in the detection of the most common EGFR mutations in Caucasian lung adenocarcinoma, comparing the immunohistochemical results with the EGFR mutational status, detected by pyrosequencing.

In addition, the expression of the mutant protein and the mutation status of EGFR were related with clinico-pathological data of each tumor and with patient's outcome.

3. MATERIALS AND METHODS

3.1 Patients and Samples

The subjects of this study were 117 Caucasian patients with lung adenocarcinoma histologically diagnosed at the Department of Surgical and Morphological Sciences, Unit of Pathology, University of Insubria, between 1996 and 2013. Particularly the study included 89 cases with stage I (TNM 2010) primary lung adenocarcinomas who underwent surgical resection at the Center for Thoracic Surgery of the University of Insubria, Varese Hospital, between February 1996 and October 2006, and 28 cases, 19 surgical specimen and 9 needle biopsies (6 pulmonary biopsies, 2 cerebellum metastasis biopsies and 1 pleural biopsy) obtained from patients in advanced stage disease, who were clinically selected for tyrosine kinase inhibitor (TKI) administration, between 2010 and 2013. The selection of the cases was based on the availability of sufficient tumor samples for histopathological, immunohistochemical, and molecular analysis.

Clinical and pathologic data were obtained by reviewing medical records and pathology reports.

All tumours were reviewed and histological classification was determined on hematoxylin and eosin-stained sections according to the most recent International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) classification (Travis WD, 2011) and using immunohistochemical panel including markers of squamous (CK5/6, CK14 and p63) and glandular (TTF-1, SPA, CK7 and CK18) cell differentiation and when necessary, the original classification was changed. The degree of differentiation (well, moderate and poor) was assessed according with WHO lung tumors classification (Travis, 2004). The pathologic staging was determined according to the TNM seventh edition recommendations (Goldstraw P, 2010).

3.2 Mutation-specific antibodies and immunohistochemical study

For each patient, the immunohistochemistry (IHC) was performed on the same histological sections obtained from the same paraffin blocks used for pyrosequencing.

Paraffin sections were collected on Superfrost Plus slides and completely processed automatically with a BenchMark XT immunostainer (Ventana) using the optiview DAB detection kit (Ventana). The mutation-specific antibodies were the ready-to-use rabbit monoclonal anti-EGFR E746-A750 del (clone SP111, Ventana Medical Systems, Inc.) and anti-EGFR L858R (clone SP125, Ventana Medical Systems, Inc.) antibodies. In particular, antigen retrieval was performed at 95°C for 28 min (for SP111) and 24 min (for SP125), and the primary antibody was applied at 37°C for 28 min and 24 min for SP111 and SP125, respectively.

Immunoreactivity was evaluated and recorded as percentage of positive cells (0-100%) on the total of neoplastic population and on the basis of the cytoplasmic and/or membrane-staining intensity, as follows: no or weak staining intensity in < 10% of tumor cells (0); weak staining in

≥10% of tumor cells (1+); moderate staining in ≥10% of tumor cells, (2+); and intense staining in ≥10% of tumor cells (3+). IHC staining was independently assessed by two pathologist (FF and ACH).

3.3 EGFR mutation analysis

Genomic DNA was extracted after manual micro-dissection from four 8µm slides, using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany).

EGFR mutation analysis was performed in duplicate by pyrosequencing using EGFR TKI response (sensitivity) kit (Diatech, Jesi, Italy), according to the manufacturer's instructions. In brief, EGFR exons 18 to 21 were amplified using Rotor Gene instrument (Corbett Research, UK) and sequenced using the PyroMark Q96 ID system (Qiagen).

Limit of detection studies for pyrosequencing were performed by a titration assay using DNA samples from 2 EGFR mutated cancer cell lines (NCI-H1975, HCC-827) and A549 cell line for wild-type DNA. To assess the analytic sensitivity, different amounts of mutant or wild-type DNA were tested in PCR reactions (50, 10, 5, 0.5, and 0.1 ng).

Titration assay showed that pyrosequencing was able to identify a mixture containing 9% of mutant alleles for E746-A750del and 7.5% for L858R mutation, respectively. Finally, input mass titration experiments demonstrated that the EGFR mutation status was reproducibly detected in 0.5 to 50 ng of tumor DNA.

3.4 Statistical analysis

To evaluate the concordance between immunohistochemical and molecular results, sensivity, specificity, positive predictor value (PPV), and negative predictor value (NPV) were calculated. The EGFR mutation status was correlated with clinico pathologic characteristics using chi-square test or Fisher's exact test.

Statistical significance was assumed for a *p* value lower than 0.05.

4. RESULTS

4.1 Clinico-pathological data

Of the 117 patients, 81 (69.3%) were men, and 36 (30.7%) were women. The mean age was 65 years (range: 36-79 years); on the whole study population, 66 years for males (range: 48-79 years) and 63 years for females (range 36-75 years).

The smoking history was available in 102 patients. Among them 88 patients (86.3%) were current or former smokers (52 and 36 respectively) including 19 (21.6%) females and 69 (78.4%) males, and only 14 (13.7%) were never smokers, including 11 females (78.6%) and 3 males (21.4%).

Of the 117 cases, 108 patients underwent radical anatomical resection, (106 by lung lobectomy and 2 by pneumonectomy) and 9 patients were subjected to needle biopsy.

The median tumour diameter was 2.8 cm (range: 0.5-7.5 cm).

Postoperative pathological staging showed 93 (86.1%) patients with stage I disease (48 stage IA and 45 stage IB), 3 (2.8%) stage II patients (2 stage IIA, and 1 stage IIB) and 12 (11.1%) patients with IIIA stage disease.

According to IASLC/ATS/ERS classification, the most common histological subtype was acinar predominant (61 cases; 52.1%), followed by solid (24 cases; 20.5%), papillary (15 cases; 12.8%), lepidic (9 cases; 7.7%), micropapillary (6 cases; 5.1%) and mucinous (2 cases; 1.8%) predominant adenocarcinoma.

With regard to tumour differentiation, 12 cases (10.2%) were classified as well differentiated (G1), 78 (66.7%) as moderately differentiated (G2), and 27 (23.1%) as poorly differentiated (G3).

Follow-up data were available for 89 of the 117 patients with ADCs underwent surgical resection. Forty nine patients (55.1% of cases) died of disease after a median time of 47 months (range: 4-156), whereas 40 patients (44.9% of cases) were still alive after a median follow-up time of 181 months (range: 48-169).

4.2 Immunohistochemical study

The expression of EGFR mutation-specific proteins, both E746-A750 deleted (clone SP111) and L858R point (clone SP125) mutated, was evaluated in all 117 patients by immunohistochemistry.

The non neoplastic lung tissues revealed no or weak expression of mutant protein. The anti L858R antibody was faintly expressed in the cytoplasm and/or membrane of normal non-neoplastic bronchial epithelial cells but not in alveolar pneumocytes. A few alveolar macrophages, inflammatory cells, necrotic areas, and mesenchymal tissue showed weak cytoplasmic staining for the anti SP111 antibody.

The pattern of immunostaining in tumor cells was either predominantly cytoplasmic, o predominantly membranous, o both.

A total of 22 cases (18.8%) showed immunoreactivity for EGFR mutant protein.

E746-A750 del specific protein (SP111) expression was detected in 8 (36.4%) of 22 adenocarcinomas (1 needle biopsy and 7 surgical specimens), including 1 (12.5%) cases with expression score 1+, 2 (25.0%) cases with score 2+ and 5 (62.5%) cases with score 3+ (Figure 3). The mean percentage of positive cell was 30%, 50% (range: 20-80%) and 47% (range: 15-90%) in ADC with weakly, moderate and strong immunoreactivity, respectively. Based on IASLC/ATS/ERS classification, among the 8 cases of lung adenocarcinomas with SP111 expression there were 6 (75.0%) predominantly acinar, 1 (12.5%) micropapillary and 1 (12.5%) solid adenocarcinomas.

The L858R specific protein expression (SP125) was observed in 13 (59,1%) of 22 positive cases, including 1 needle biopsy and 12 surgical specimens. Six cases (46.1%) showed a low level of expression (score 1+) and 7 out 13 cases demonstrated a high level of protein expression: score 2+ in 3 (23.1%) cases and score 3+ in 4 (30.8%) cases (Figure 4). The mean percentage of positive cell was 11% (range: 10-15%) in score1+ cases, 37% (range: 20-60%) in score 2+ cases and 83% (range 70-90%) in score 3+ cases. Among the 13 cases with SP125 expression, the most common histological subtype was acinar (6 cases; 46.1%), followed by solid (4 cases; 30.8%), micropapillary (2 cases; 15.4%) and papillary (1 case; 7.7%) predominant ADC.

One case showed concomitant immunoreactivity for the two antibodies, with high levels of L858R-specific protein expression (score 3+) and low level (score 1+) of E746-A750del specific protein expression. According to morphological type, the case was a micropapillary predominant adenocarcinoma.

4.3 Molecular analysis

Pyrosequencing analysis was performed in duplicate on 61 cases, 9 needle biopsy and 52 surgical specimens. EGFR mutations were found in 19 of 61 ADCs (31.1%).

Exon 19 E746-A750 del mutation was detected in 11 out 19 (57.9%) cases whereas exon 21 L858R mutation was detected in only 6 (31.6%) cases (figure 5).

One sample displayed 2 concomitant mutation (E746-A750del/L585R) and another case showed exon 18 G719C mutation.

4.4 Correlation between the expression of mutation-specific proteins and the EGFR-mutational status

The correlation between the expression of mutation-specific proteins and EGFR-mutational status is presented in Table 2.

Of the 12 cases with E746 A750 del in exon 19, an immunohistochemical score of 3+ was observed in 5 (41.6%) cases, a score of 2+ in 2 (16.7%) cases, a score of 1+ in 1 (8.3%) case; 4 (33.4%) cases were negative. In contrast, among 49 cases without E746-A750 del in exon 19, most cases (48/49,

97.9%) were negative for the SP111 antibody; only 1 case showed immunoreactivity for specific antibody but with low level score (score 1+) of expression.

Of the 7 cases with L858R mutation in exon 21, an immunohistochemical level score of 3+ was observed in 5 (71.4%) cases and a score of 2+ in 2 (28.6%) cases.

Among 54 cases without L858R mutation, the most cases were negative (47/54; 87.1%); 7 cases showed immunoreactivity for SP125, including 6 (11.1%) cases with score 1+ and 1 (1.8%) case with score 2+.

The case with both E746 A750 del and L858R mutations, showed a high level (score 3+) of L858R specific protein expression and weak level (score 1+) of E746-A750 del specific protein expression.

The case with G719C mutation in exone 18 did not show immunohistochemical reactivity for both antibodies.

Table 2. Correlation between results of the mutation specific immunohistochemistry (IHC) and EGFR mutational status

EGFR mutation status	N°=61	IHC E746-A750 del (SP111) score level				IHC L858R (SP125) score level			
		0	1+	2+	3+	0	1+	2+	3+
E746 A750 del									
Mutated	12(19,7)	4(33.4)	1(8.3)	2(16.7)	5(41.6)	0(0)	0(0)	0(0)	0(0)
Wild type	49(80,3)	48(97.9)	1(2.1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
L858R									
Mutated	7(11.5)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(71.4)	5(28.6)
Wild type	54(88.5)	0(0)	0(0)	0(0)	0(0)	47(87.1)	6(11.1)	1(1.8)	0(0)
G719C									
Mutated	1(1.7)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Wild type	60(98.3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

Sensitivity, specificity, positive predictor values (PPV), and negative predictive values (NPV) were calculated according to each immunohistochemical score (Table 3).

For the detection of E746 A750 del by immunohistochemistry, the sensitivity, the specificity, the PPV and the NPV were 41.7%, 100%, 100% and 87.5%, respectively, considering as positive only cases with high level (score 3+) of SP111 antibody expression.

If an immunohistochemical score of 2+ was defined as the cut off point, the sensitivity was 58.3%, the specificity was 100%, the PPV was 100% and the NPV was 91%.

Considering all cases with anti-EGFR E746-A750del specific antibody expression, we observed an increase of sensitivity and a reduction of the specificity (66.7% and 98.0% respectively); the PPV was 88.9% and the NPV was 94.2%.

For the detection of L858R in exon 21 by SP125 antibody, the sensitivity was 71.0%, the specificity was 100%, the PPV was 100%, and the NPV was 96.4%, if an immunohistochemical score of 3+ was defined as the cut off point.

Considering as positive the cases with moderate (score 2+) and high (score 3+) level of SP125 antibody expression, the sensitivity, the specificity, the PPV and the NPV were 100%, 98.1%, 87.5% and 100%, respectively.

We observed a reduction of the specificity (87.0%) and the PPV (50.0%), considering all cases with anti-EGFR L858R specific antibody expression. The sensibility and the NPV were 100%.

Significantly correlation with EGFR-mutational status and immunohistochemical expression was showed in cases with a score ≥2+ for SP111 antibody and in cases with a score ≥3+ for SP125 antibody.

Table 3. Diagnostic power of mutation-specific antibodies comparing with EGFR mutational status

EGFR mutation specific antibodies	Score	Sensitivity (%)	Specificity(%)	PPV(%)	NPP(%)
Anti EGFR E746 A750del (SP111)	≥3+	41.7%	100%	100%	87.5
	≥2+	58.3%	100%	100%	90.7%
	≥1+	66.7%	98.0%	88.9%	94.2%
Anti EGFR L858R (SP125)	≥3+	71.0%	100%	100%	96.4%
	≥2+	100%	98.1%	87.5%	100%
	≥1+	100%	87.5%	50.0%	100%

4.5 Association between EGFR mutational status and the clinical-pathological data

The correlation between the EGFR-mutational status and the clinico pathological data is presented in Table 4.

Among the 19 ADCs with EGFR mutations, there were 14 (73.7%) females and 5 (26.3%) males (p=0.0005).

The mean age of patients with EGFR mutations (68 years; range: 53-75) was older than the mean age of EGFR wild type patients (64 years; range: 48-79) (p=0.053), without significantly differences between women and men.

The smoking history was available in 55 of 61 patients, including 45 current or former smokers and 10 never smokers. EGFR mutations were significantly more frequent in never smokers (6/10;

60%) than in current or former smokers (8/45; 17.8%)(p=0.011). Most of EGFR wild type cases were observed in current or former smokers cases and in male patients (p= 0.045).

Among the 15 patients with EGFR mutations who underwent radical anatomical resection, the median tumor diameter was 2.9 cm (range: 0.8-5.5 cm).

EGFR mutations were more frequent in ADCs with well or moderately differentiated tumors (18/19; 94.7%) than in poorly differentiated ADCs.

Based on IASLC/ATS/ERS adenocarcinoma classification, EGFR mutations were more frequently found in the ADC with acinar (14/19; 73.7%) (p=0.05) and micropapillary (4/19; 21.0%) (p=0.043) histological pattern. One case (1/19; 5.3%) was lepidic predominant adenocarcinoma. EGFR mutations were not detected in solid (p=0.01), mucinous and papillary adenocarcinomas.

Among EGFR wild type cases, the most frequent histological subtypes were acinar (19/42; 45.2%), solid (11/42; 26.2%) and papillary (6/42; 14.3%) predominant ADCs. Three cases (7.1%) were lepidic type, 2 (4.8%) cases were mucinous type and 1 (2.4%) case was micropapillary type ADC.

Exon 19 E746-A750 del mutation was more frequently observed in females (9/11; 81.8%), and in well or moderately differentiated ADCs (11/11; 100%). Ten cases (90.1%) were acinar type, while only 1 case was micropapillary type. The mean age was 69 years (range: 53-74 years).

Exon 21 L858R mutation was detected in 6 cases. The mean age was 66 years (range: 57-74 years). The majority of cases were well differentiated (5/6; 83.3%) and with acinar predominant type (4/6; 66.7%). No differences were observed among the two gender.

The exon 18 G719C mutation was observed in a 62 year old woman, without smoking history and lepidic predominant ADC type.

Concomitant expression of both mutation (E746-A750 del/ L858R) was observed in a 73 year old woman, without smoking history and with moderately differentiated micropapillary type ADC.

4.6 Association between EGFR mutational status and the clinical outcome

Follow-up data were available for 40 of 61 patients in which the molecular analysis was performed. No significant association was found between EGFR mutational status and patients outcome.

However, median survival was longer in the 32 patients with EGFR wild type ADCs than in the 8 patients with EGFR mutated ADCs (70 months vs. 56 months). Among EGFR mutation group, three patients (75% of cases) died of disease after a median time of 45 months (range: 16-89), whereas 4 patients (25% of cases) were still alive after a median follow-up time of 64 months (range: 49-76).

Table 4. Correlation between EGFR mutational status and the clinical-pathological data

Clinicopathologic features	N=61	EGFR mutation status		p value
		Mutated (%)	Wild type (%)	
Gender				
Male	37	5 (13.5)	32 (86.5)	0.0005
Female	24	14 (58.3)	10 (41.7)	
Age				
>65 yr	33	14 (42.4)	19 (57.6)	0.053
≤ 65yr	28	5 (17.9)	23 (82.1)	
Smoking history#				
Active-former smokers	45	8 (17.8)	37 (82.2)	0.0117
Never smoker	10	6 (60.0)	4 (40.0)	
Tumor diameter*				
≤2,8 cm	36	11 (30.6)	25 (69.4)	0.215
>2,8 cm	16	4 (25.0)	12 (75.0)	
Grade of differentiation				
Well-moderately	51	18 (35.3)	33 (64.7)	0.015
differentiated	10	1(10.0)	9 (90.0)	
Poorly differentiated				
Histology				
Acinar	33	14 (42.4)	19 (57.6)	0.052
Lepidic	4	1 (25.8)	3 (74.2)	
Papillary	6	0 (0)	6 (100)	0.16
Micropapillary	5	4 (80.0)	1 (20.0)	0.043
Solid	11	0 (0)	11 (100)	0.011
Mucinous	2	0 (0)	2 (100)	
Pathologic stage*				
IA	20	4 (20.0)	16 (80.0)	
IB	23	5 (21.7)	18 (78.3)	
IIA	1	0 (0)	1 (100)	
IIIA	8	6 (75.0)	2 (25.0)	

#Not available: 6 patients
*9 cases were needle biopsy

Figure 3. Immunohistochemical staining with EGFR del E746-750 mutation specific antibody (SP111) in lung adenocarcinoma. **A**: high intensity (score 3+), x200 magification; **B**: moderate intensity (score 2+), x400 magification; **C**: low intensity (score 1+), x400 magification.
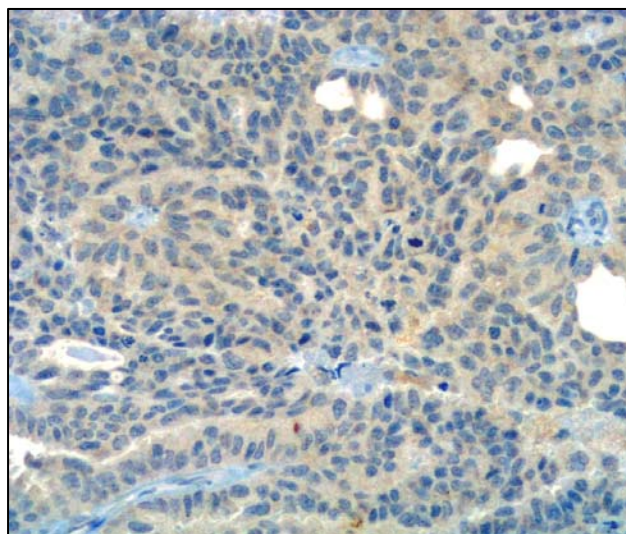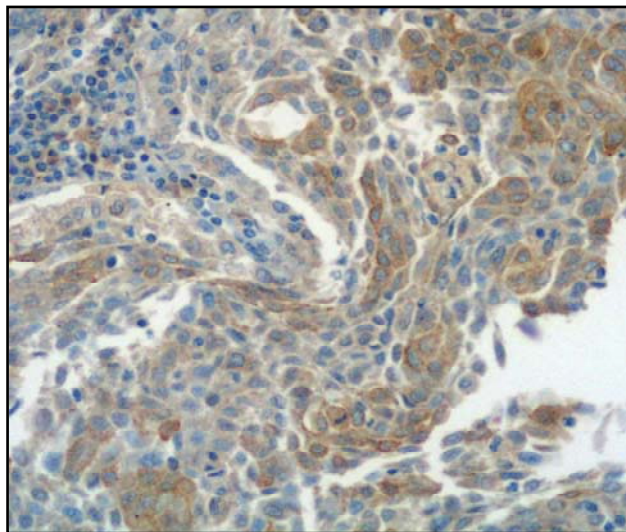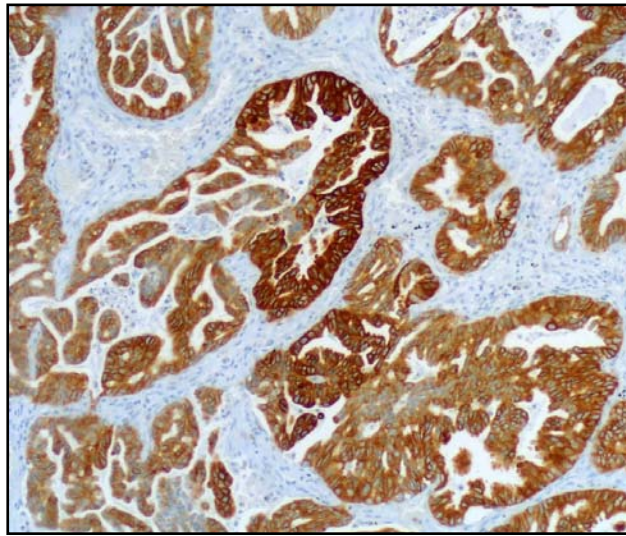
Figure 4. Immunohistochemical staining with L858R mutation-specific antibody (SP125) in lung adenocarcinoma. **A**: high intensity (score 3+), x200 magification; **B**: weak intensity (score 1+), x400 magification.
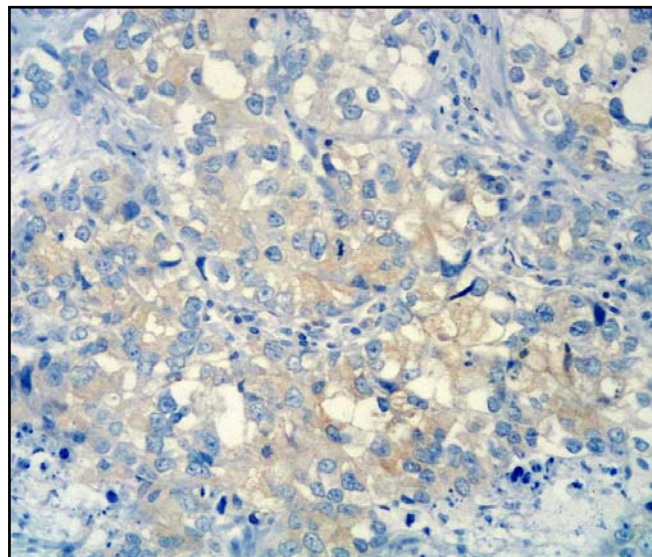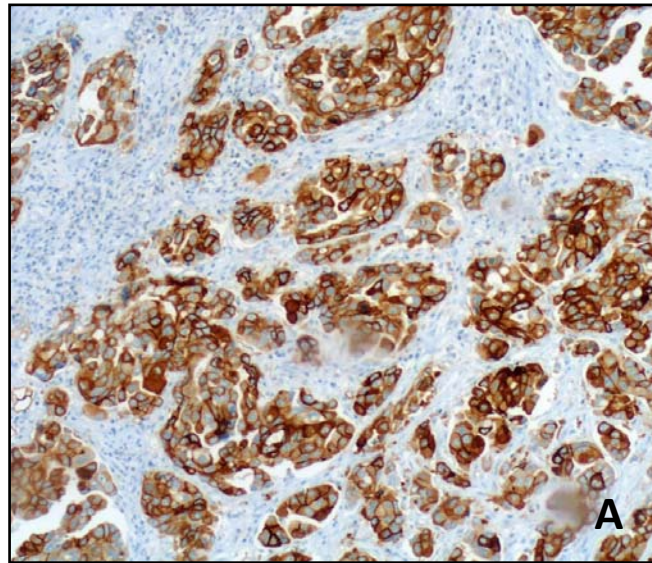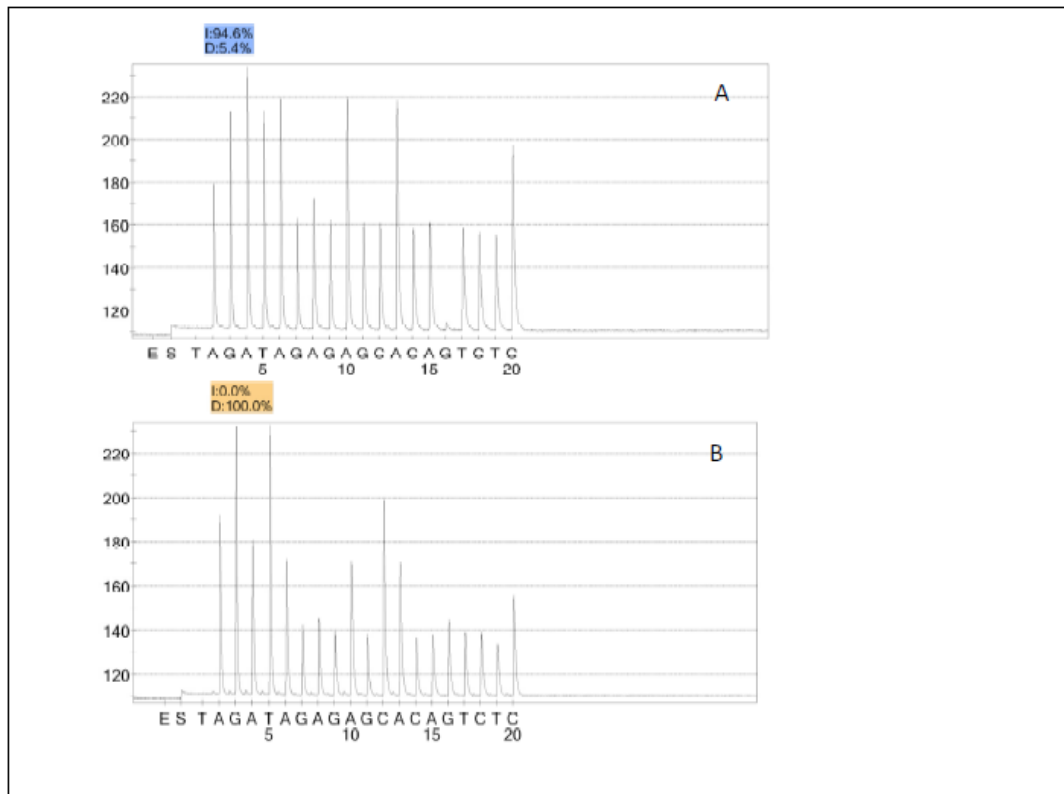
Figure 5. Pyrosequencing analysis. **A**: exon 19 wild type; **B**: exon 19 E746-A750 del mutation.

5. DISCUSSION

Primary lung cancer is the most common cancer and the leading cause of the death from malignant tumors worldwide (Siegel R, 2013).

About 85% of all lung cancer patients have non-small cell lung cancer (NSCLC), and the majority presents with advanced stage disease at the time of diagnosis. The standard of care for these patients is platinum-based combination chemotherapy. This strategy results in a median overall survival of 8 to 10 months and a 1 year survival rate of about 33% (Azzoli CG, 2009). In the last years new therapeutic options to improve outcomes from this disease have been developed. The dominant topic of such investigations has been aimed toward identifying specific molecular targets to develop new agents with the capacity to kill cancer cells with minimal toxicity to normal cells.

Presence of activating mutations in epidermal growth factor receptor (EGFR) predicts response to EGFR tyrosine kinase inhibitors, gefitinib and erlotinib, in patients with advanced or recurrent non-small-cell lung cancer (NSCLC). Therefore, EGFR mutational status should be determined before initial treatment (Tsao MS, 2005; Mitsudomi T, 2010; Fukuoka M, 2011).

In frame deletion in exon 19 (E746-A750del) and the single point mutation L858R in exon 21 account for approximately 90% of the drug sensitive EGFR mutations and are associated with responsiveness and prolonged survival in NSCLC (Lynch TJ, 2004; Pao W, 2004; Sharma SV, 2007).

Other important mutations are insertions in exon20 (T790M), which predicts resistance to EGFR tyrosine kinase inhibitors, and activating mutations of exon 18 (G719AC) and exon21 (L861Q). Several other EGFR mutations have been reported, but their clinical significance is yet unknown.

Mutation rate in adenocarcinomas in East Asian patients are 20-50% (Thunnissen E, 2012) and 5-15% in Western European patients depending on whether selected or unselected patients are tested (Sakurada A, 2011; Tsao MS, 2011; Skov BG, 2014).

Small biopsies or cytological specimens may be the only diagnostic materials available for EGFR mutation testing in those patients, so accurate methods to detect mutational status would have great clinical import.

No gold standard method for the detection of EGFR mutations in NSCLC is approved. Sanger DNA sequencing has been widely used, but its disadvantages, primarily its low sensitivity (requirement of 40-50% mutant DNA in samples), has led to the development of more sensitive detecting methods including pyrosequencing (Sahnane N, 2013). However, these methods are relatively

expensive, time consuming, and not incorporated in routine diagnostic procedures in many departments of pathology. In contrast, immunohistochemistry (IHC) has lower costs, shorter turnaround time, and is available in the majority of laboratories. For these reasons, mutation-specific antibodies might be a relevant alternative for determining EGFR status in NSCLC.

The accuracy of mutation-specific antibodies has been evaluated in several studies. These works used different scores for intensity and cut off point for positivity making comparisons between the individual studies difficult. So far, no standardized protocol exists for EGFR immunohistochemistry in NSCLC.

In this study, the accuracy of two recently developed mutation-specific antibodies, SP111 for E746-A750 del and SP125 for L858R, was evaluated and compared with the EGFR-mutational status (Table 2. and Table 3.).

Mutant EGFR protein expression was present in 22 cases (36.1%); 8 cases were positive for SP111 and 13 cases were positive for SP125. EGFR mutations were found in 19 of 61 ADCs (31.1%). Exon 19 E746-A750 del mutation was detected in 11 out 19 (57.9%) cases whereas exon 21 L858R mutation was detected in only 6 (31.6%) cases.

In our work, specificity of the mutation-specific antibodies in detecting deletions in exon 19 and mutations in exon 21 was high. Using an immunohistochemical score 3+ as a criterion of positivity, each antibody had a specificity of 100% for E746 A750 del and L858R and the PPV was 100%. Considering as positive the cases with moderate (score 2+) and high (score 3+) levels of antibody expression, both the specificity and the PPV were 100% using SP111, while they lowered to 98.1% and 87.5%, respectively, using SP125.

The high specificity observed for both antibodies is in line with the literature, where specificity for exon 19 mutations ranged from 92 to 100% and for exon 21 mutations from 97 to 100%, albeit these studies employed different mutant-specific antibodies (clone 6B6 and clone 43B2) compared to the ones used in our work (Yu J, 2009; Brevet M, 2010; Kato Y, 2010; Kawahara A, 2010; Kitamura A, 2010; Ilie MI, 2010; Nakamura H,2010, Simonetti S, 2010; Kozu Y,2011, Bondgaard AL, 2014).

In a recent report, Seo et al (Seo AN, 2014), using the same antibodies employed in the present study, found a specificity of 99.0% for SP111 and 89.7% for SP125 with a cut off score ≥2+. The specificity and the PPV for both antibodies were 100% with immunohistochemical score 3+.

In contrast to the high specificity, in our study the sensibility of the mutation-specific antibodies was low, particularly for exon 19 deletion.

The sensitivity, considering as positive only cases with high levels (score 3+) of SP111 and SP125 expression, was 41.7% for E746 A750 del and 71.0% for L858R. Lowering the positivity cut off to 2+ or 1+ would improve the sensivity but could result in false positive interpretations.

This finding is in agreement with the literature. Previous studies using the mutation-specific antibodies (clone 6B6 and clone 43B2) have found sensitivity ranging from 23 to 80% for exon 19 deletions and 36 to 100% for exon 21 mutations (Yu J, 2009; Brevet M, 2010; Kato Y, 2010; Kawahara A, 2010; Kitamura A, 2010; Ilie MI, 2010; Nakamura H,2010, Simonetti S, 2010; Kozu Y,2011, Bondgaard AL, 2014). Seo et al (Seo AN, 2014) found a sensivity of 29.4% for SP111 and 41.3% for SP125, using an immunohistochemical score 3+.

In our study four false negative cases for E746-A750 del (immunohistochemistry negative, molecular analysis positive) were observed, including a needle biopsy and three surgical specimens. In 2 cases the mean percentage of allelic mutation was low (22% and 25%) suggesting that a positive reaction for mutation-specific antibodies may be affected by the EGFR gene copy number. Mass spectrometry based genotyping (Sequenom) was performed on one case, which revealed a minor variant of exon 19 del (p.746_S5752>V). Therefore, the low sensitivity for exon 19 deletions is mostly explained by the fact that the exon 19 antibody only detects the most common deletion of exon 19 (deletion E746-A750) but not detected a part of its minor variants. In the study of Kitamura et al. (Kitamura A, 2010;) the authors suggested that clone 6B6 detected only E746-A750 del and its minor variants such as E746-A750>K (9.5%), whereas E746-A750>RP and L747-A750>P were not detected. Deletion E746-A750 is of 15 base pairs and represents 50-65% of all exon 19 deletions. However, deletions of 9, 12, 16, 18, and 24 base-pairs have been identified, each producing slightly different epitope not detectable by the exon 19 antibody (Yu J, 2009). Previous studies using clone 6B6 for E746-A750 del demonstrated that the sensitivity of clone 6B6 for non-E746 A750 del varied considerably depending on the deletion size, and ranged from 20% to 67% (Brevet M, 2010).

Therefore, in patients with negative immunohistochemical result against E746-A750 del, further molecular testing should be performed to exclude false negative results or other variants of 19 del.

In this study a case showed exon 18 G719C mutation, but it did not show immunohistochemical reactivity for any of the two antibodies.

Immunohistochemistry (IHC) has well known advantages as a testing method, in comparison with molecular assays. IHC saves time, is cost-effective, and can be performed in most pathology laboratories. Another advantage of IHC over molecular techniques is that it can distinguish between tumor morphology and mutation-bearing cells by light microscopy. This technique, however, has some disadvantages including generally higher interlaboratory variability in assay performance and a certain degree of interobserver variability in assay interpretation. These aspects of IHC lead to a higher risk of false positives than molecular assays, unless staining is scored in a consistent and rigorous fashion.

In our works the specimens were completely processed automatically with an immunostainer with the aim of reducing the variability in performance.

In this work, the case with a score 3+ both for E746-A750 del that for L858R, showed excellent inter observer agreement; moreover, immunohistochemical scores 3+ completely correlated with the EGFR-mutational status. This results suggest that patients with an immunohistochemical score of 3+ (specificity of 100% and the PPV of 100%), also with a low percentage of positive cells, might be potential candidates for EGFR-TKIs treatment.

However, by the same token, in patients with an immunohisochemical score of ≤2, confirmative molecular testing would appear essential to obtain reliable EGFR mutation results, in order to exclude false positive results.

A further consideration is that these IHC antibodies obviously cannot detect other clinically relevant EGFR mutations, such as exon 18 G719 point mutations, or exon 20 mutations, including the T790M resistance mutation.

A second point of our work regards the association of EGFR mutation status with clinico-pathological data (Table 4.). In this study the incidence of EGFR mutations in lung adenocarcinomas was 31.1% (19 of 61 cases).

The incidence of EGFR mutations in lung adenocarcinoma is associated with gender and smoking history. In our study we confirmed that EGFR mutation was more frequent in female (p=0.0005) and in never smokers (p=0.0117). Past report suggested the impact of age on EGFR mutation, and concluded that age was associated with EGFR mutation in lung cancer (Ueno T, 2012; Zhang Y, 2012). In this study the mean age of patients with EGFR mutations (68 years versus 64 years) was higher than the mean age of EGFR wild type patients (p=0.053), and this finding is in agreement with result reported.

EGFR mutations were more frequent in well or moderately grade of differentiated ADCs than in poorly differentiated ADCs (p=0.015), in agreement with data reported in literature (Liu Y, 2009).

The present study, in agreement with previously reports (Shim HS, 2011; Russell PA, 2013) showed that the frequency of EGFR mutations was different between histological subtypes of lung adenocarcinoma. EGFR mutant tumors were more likely acinar and micropapillary predominant ADCs (p=0.043). None of the EGFR mutated cases were solid or mucinous adenocarcinomas. Classification of the sub-histology of lung adenocarcinoma in small biopsy specimens is relevant because particularly subtypes were associated with EGFR mutations and it provides prognostic information.

In conclusion, IHC assays using EGFR mutation specific antibodies against del E746-A750 and L858R have a very high specificity in identifying EGFR mutations, but did not show high sensitivities compared to the highly sensitive molecular method. IHC is a useful screening method for detecting EGFR mutation in most pathology laboratories, not only for resection samples but also for biopsy cases, and can quickly identify candidates for EGFR-TKI therapy.

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