Somatic genomic alterations in lung adenocarcinoma: non-invasive molecular diagnosis and prognosis impact of driver mutations in non-tumoral airway cells.

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SUMMARY

Lung adenocarcinoma is the most prevalent histological subtype of lung cancer and regardless of the major efforts in prevention policies, early detection and research, the overall 5-year survival is less than 17%. Surgical resection is the first-line treatment for early-stage ADC, but tumor recurrence is frequent and survival rates remain low comparing with other neoplasms. Lung ADC has a high mutational burden and somatic mutations can be found in more than 75% of the cases with a high proportion of oncogenic driver alterations that have potential therapeutic implications. These characteristics force us to make a wide molecular diagnosis in all our patients with ADC in order to offer therapeutic alternatives. To date, pathological samples are required to perform the molecular study while cytological samples are considered to have limited utility. We hypothesized that carcinogenic factors will promote loco-regional modifications not only in the future tumor, but throughout the exposed lung, and that these genomic alterations can be identified in cytological specimens obtained by non-invasive endobronchial techniques. This PhD thesis is composed by three original manuscripts and two complementary papers.

Manuscript I: our hypothesis was that carcinogenic factors would promote genomic changes not only in the ADC tumor but also in the exposed lung, and this could be related with prognosis. We have observed 21.3% of patients with *EGFR* or *KRAS* mutations in their ADC tumor show the same alterations in histologically normal lung tissue. Moreover, their 12-months prognosis is worse than that of subjects without this finding.

Manuscript II: we hypothesized that performing mutational analysis with brushing specimens obtained by radial endobronchial ultrasound (R-EBUS) plus fluoroscopy-guided bronchoscopy in patients with peripheral pulmonary ADC was feasible. Using cytological extensions conserved in RPMI culture medium we could isolate DNA and perform molecular analysis in 100% of the patients. We also found a correlation of 86.6% in the detection of *EGFR* and *KRAS* in histological and cytological samples.

Manuscript III: this study pretended to confirm the hypothesis of the first manuscript and demonstrate the presence of *EGFR* or *KRAS* mutations in non-tumoral lung cells in patients with localized adenocarcinoma but with negative genomic testing in the tumor. We confirmed the presence of mutations in 9.7% of the cases. With this study we demonstrate that the presence of the mutations in the lung were not secondary to circulating tumoral cells or tumoral DNA.

Two complementary thesis manuscripts were also published, one of them is an exhaustive **review** of the etiopathogenesis of adenocarcinoma and its molecular alterations, and the other an **editorial** of how the immune phenotypes in lung cancer patients with COPD could have potential implications in immunotherapy

RESUMEN

El adenocarcinoma pulmonar (ADC) es el tipo de cáncer de pulmón más frecuente. A pesar de los grandes esfuerzos en prevención, detección precoz e investigación, la supervivencia a 5 años es inferior al 17%. La resección es el tratamiento de elección en estadios iniciales, pero las recurrencias son frecuentes y el pronóstico sigue siendo malo comparado con otras neoplasias. El ADC tiene una alta carga mutacional y en más del 75% de los casos se pueden detectar alteraciones moleculares somáticas, de las cuáles una alta proporción están en oncogenes ("driver mutations") y tienen potencialmente implicaciones terapéuticas. Esto obliga en todos los pacientes a hacer un diagnóstico molecular amplio para ofrecer alternativas terapéuticas. Las muestras patológicas son de elección para el diagnóstico molecular mientras que las muestras citológicas parecen tener una utilidad limitada. Nuestra hipótesis es que los factores carcinogénicos afectan a todas las células expuestas y podrían producir alteraciones genómicas no solo en el tumor, sino en otras células aparentemente sanas del pulmón. Estas alteraciones se pueden identificar en muestras citológicas obtenidas con técnicas endoscópicas no invasivas. Esta tesis doctoral está compuesta por tres manuscritos originales y dos artículos complementarios.

Artículo I: nuestra hipótesis es que las mutaciones somáticas ocurren no solo en células tumorales sino también en pulmón sano, pudiendo asociarse con un peor pronóstico. En el 21.3% de los pacientes encontramos la misma mutación, *EGFR* o *KRAS*, en el tumor y en tejido pulmonar sano. Este hallazgo condiciona además un peor pronóstico a los 12 meses.

Artículo II: nuestro objetivo fue demostrar que en muestras de cepillado bronquial obtenidas mediante ecobroncoscopia radial guiada por fluoroscopia, se puede realizar el estudio genómico del tumor en pacientes con ADC. Utilizando muestras citológicas preservadas en medio RPMI (Roswell Park Memorial Institute), se pudo realizar el análisis molecular en el 100% de los casos, mostrando una correlación del 86.6% en la detección de *EGFR* y *KRAS* entre muestras histológicas y citológicas.

Artículo III: este estudio pretende confirmar la hipótesis del primer artículo al demostrar alteraciones en el *EGFR* o *KRAS* en pacientes con ADC sin mutaciones en el tejido tumoral. Confirmamos que en un XX% de los pacientes existen mutaciones en el tejido sano independiente de las mutaciones en el tumor, lo que demuestra que dichas alteraciones no son secundarias a células o DNA tumoral circulante

Dos artículos complementarios se anexan también en esta tesis. El primero es una **revisión** exhaustiva de la etiopatogenia del ADC, mientras que el segundo se trata de un **editorial** acerca de cómo los fenotipos inmunológicos en pacientes con EPOC y cáncer pueden tener implicaciones en la inmunoterapia.

PREFACE

This PhD thesis has been developed and organized in order with the policy approved by the Doctoral Committee of the Universitat Pompeu Fabra. All the published and no-published data have been obtained in accordance with the ethical guidelines of the Declaration of Helsinki and local legislations, with particular emphasis on regulations regarding data privacy and biological specimen collection.

We recognize the carcinogenesis process, especially in lung cancer, as a multi-hit and multi-step model which implies that an intrinsic susceptibility in the subject with a maintained exposition to deleterious factors, such as tobacco smoke or contamination. These factors can create an appropriate lung microenvironment and immune misbalance that will produce a clonal expansion (1). In adenocarcinoma, all this process known as "field cancerization", include the gaining of specific somatic genomic alterations in more than 70% of the cases and, with the available evidence at today, these genomic changes will necessarily produce a clonal cell expansion. Regardless of these knowledge, we also think that taking into account the high recurrence rate in patients with localized lung adenocarcinoma and a "whole-lung" cancerization effect, not only the tumoral cells will present acquired changes on its genome. This novel principal hypothesis became the basis from which all the studies that compose this thesis are derived.

The complex process of obtaining a precise diagnosis of lung adenocarcinoma in order to offer a high quality, humanized and personalized treatment to all patients, forces us to work in a coordinated and systematic approach. Specially, to be permanently at the forefront of all the new evidence that may impact on our medical practice. In this way, the molecular diagnosis is an exciting and permanently evolving area. The new guided treatments require complex molecular tests and demand us to obtain larger samples with a non-invasive approach. In this regard, an important part of the present doctoral thesis was to demonstrate that we could perform broad molecular studies effectively with cytological samples.

In the design process of a PhD thesis with such an ambitious aim, we needed to conform a multidisciplinary team which included pulmonologists, thoracic pathologists, thoracic surgeons, oncologists, nurses and molecular biology specialists. This was a challenge but also allowed us to obtain a solid prospective cohort, well characterized and with a strict follow-up. Likewise, it has allowed us to strengthen our serum and tissue bio-bank and to create a concrete new line of research in our department. The results of a previous pilot, not published, study using the tissue bank of the Instituto Carlos III from the Spanish Ministry of Economy, Industry and Competitiveness, motivated us to continue with this large research project.

At meantime, during the development of this research line, I have improved my skills in advanced endoscopy while being part of the respiratory endoscopy section and the lung cancer unit. Simultaneously, with the progress of the core work of this thesis, I have led several research projects especially in the area of COPD and infectious diseases, which has allowed me to improve my research skills and especially to learn how to write and review research manuscripts.

The principal issue in this whole process has been to have the opportunity to develop translational projects that potentially may have relevant impact on the knowledge we have in lung cancer, and especially, on patient's care. At the end of everything, our profession and research have as ultimate and main goal, to help those who need it, patients.

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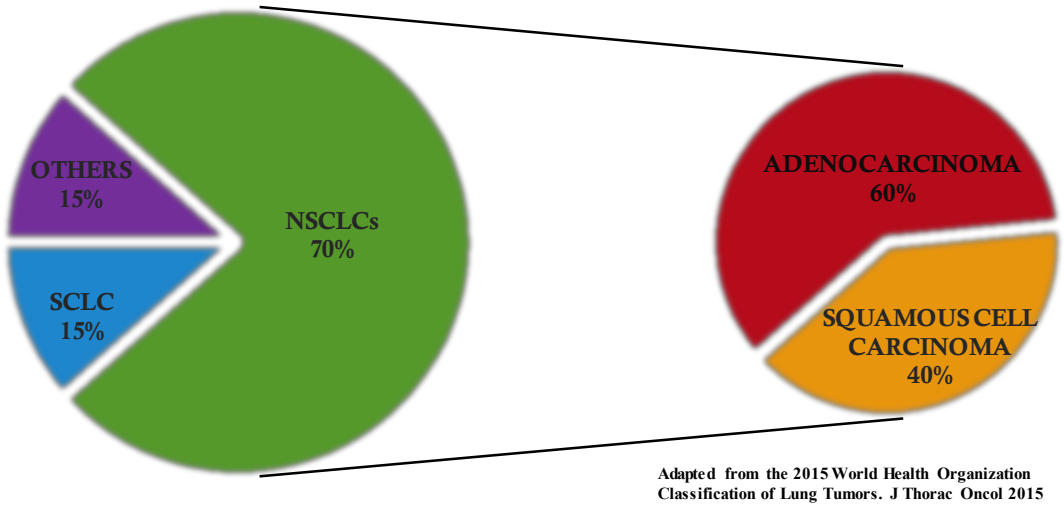
Chapter 1
Introduction

Lung cancer is considered an important public health-care issue worldwide because of its high incidence, mortality and costs. Actually, it is the second most commonly diagnosed tumor and the leading cause of cancer-related deaths in both men and women worldwide. In Spain, lung cancer is the third most frequent neoplasm with more than 28,000 new cases every year and produce 37% more deaths when compared to colorectal cancer, which is the most frequent (2,3). In the United States it represents 14% of all neoplasms and is estimated to have produced more than 150,000 deaths in the last year (4). This epidemic burden began around the mid-20th century, when the mass-production of packet cigarettes became extended in Western Europe and the United States. Tobacco smoke is the main factor for lung cancer, since it is accepted that it accounts for 80% in males and at least 50% in females. However, although the etiological role of tobacco is crucial, up to 25% of lung cancer presents in people that have never smoked. This is especially evident in women with the adenocarcinoma (ADC) subtype. In these cases, other risk factors such as air pollution, environmental and work related carcinogens also seem to play an important role (5–7). There are two main histological types of lung tumors: small-cell lung cancer (SCLC), and non-small-cell lung cancers (NSCLCs). The latter represents 80-85% of these tumors, and different histological subtypes can be distinguished: squamous cell carcinoma (SQCC) (44% in men and 25% in women), pulmonary ADC (28% in men and 42% in women) and large-cell and undifferentiated carcinomas (around 9% in both genders); rare subtypes accounting for less than 1% of the cases. However, dominant histological type strongly varies depending on the smoking status, ethnic background and geographic location, but nowadays it is accepted that the most frequent is ADC, especially in Asian women (more than 70% in Japanese females) (8,9).

Figure 1. Histological Types of Lung Cancers.



Adapted from the 2015 World Health Organization Classification of Lung Tumors. J Thorac Oncol 2015

Even though the incidence rate of lung cancer has been declining in men since the 1980s and in women since the mid-2000s, and that major efforts have been made in research, smoking prevention, early detection and global healthcare approaches, there have still been no overall significant changes in 5-year survival in the last three decades. Moreover, the 1- and 5-year survival rates in lung cancer are 44% and 17%, respectively, and even in patients with a very early stage disease, when supposedly curative surgery is performed the 5-year survival is less than 60% (10,11).

The use of next-generation sequencing (NGS) technologies has confirmed the prevalence of somatic driver alterations in more than 70% of pulmonary ADC. In fact, the Cancer Genome Atlas (TCGA) has identified that 35% of patients have mutations in oncogene *TP53* (tumor suppressor gene 53), overlapping with oncogenic driver alterations such as mutations in *KRAS* (Kirsten rat sarcoma viral oncogene), *EGFR* (epidermal growth factor receptor 1 or *ErbB1* tyrosine kinase receptor oncogene, also denominated *ErbB1* or *HER1*), *BRAF* (v-Raf murine sarcoma viral oncogene), *MET* (mesenchymal-epidermal transition oncogene, encoding a tyrosine kinase receptor), *ERBB2* (epidermal growth factor receptor 2 oncogene, also encoding a tyrosine kinase receptor, called ErbB2 or HER2 as well), and fusions in *ALK* (anaplastic lymphoma kinase), *ROS1* (encoding tyrosine-protein kinase ros) or *RET* ('rearranged during transfection', codifying a tyrosine kinase receptor) oncogenes, all of them with potential therapeutic implications. Moreover, in recent years new guided therapies have already appeared that are modifying the prognosis of selected groups of patients who have somatic driver alterations. In contrast with ADC, although *TP53* mutations are reported in as much as 81% of SQCC, targetable driver somatic alterations are not frequently found in this tumor subtype (12,13).

Chapter 2
Cancer Biology

2.1 SMOKING AND LUNG CANCER

Smoking is the most relevant risk factor to develop cancer and specially lung cancer. Tobacco has 55 compounds identified as carcinogenic to human beings and are divided into 8 carcinogen classes: polycyclic aromatic hydrocarbons, aza-arenes, n-nitrosamines, aromatic amines, heterocyclic amines, aldehydes, miscellaneous organic compounds and inorganic compounds. Of these, 20 have proven to cause lung cancer in animal models and humans. The most important groups are the polycyclic aromatic hydrocarbons and the tobacco-specific nitrosamines, specially two of last: N′-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (14,15).

The first studies demonstrating the negative effects in human health of tobacco smoking were published in the mid20th century just after the expansion of cigarette production and consumption in the World War I and World War II. The most important, "the British Doctor´s Study", was started in 1950 in the UK by Richard Doll and Austin Bradford Hill. This prospective cohort study was designed to demonstrate the association between smoking and death, included 34,439 male doctors with a final follow up of 60 years and the preliminary results were published in 1954. The study confirmed a rise in the mortality of smokers due to lung cancer as the quantity of tobacco consumption increases and all the evidence published related to this study during the next 60 years were consistent with the preliminary report (16–19). Simultaneously in the US, the veterans study, surveyed 250,000 US veterans asking for tobacco use in 1950s. The first results of this cohort were published in 1964 and the final report of a 26-year follow-up was done in 1995, demonstrating a RRs for lung cancer of 11.7 in smokers (20,21).

2.2 GENETIC RISK FACTORS

Tobacco smoking is well known as the main risk factor for lung cancer, but there is also an important percentage of never-smokers who also develop this malignancy. For instance, in the USA, 17,000-26,000 annual deaths can be attributed to lung cancer in never smokers. Thus, environmental carcinogens also seem to play an important role. These external factors appear to be combined with genetic susceptibility. In this regard, studies performed both in smokers and never-smokers strongly suggest that polymorphisms in genes involved in DNA repair, cell-cycle regulation, apoptotic pathways, inflammation and telomere length are related with lung cancer (22–25).

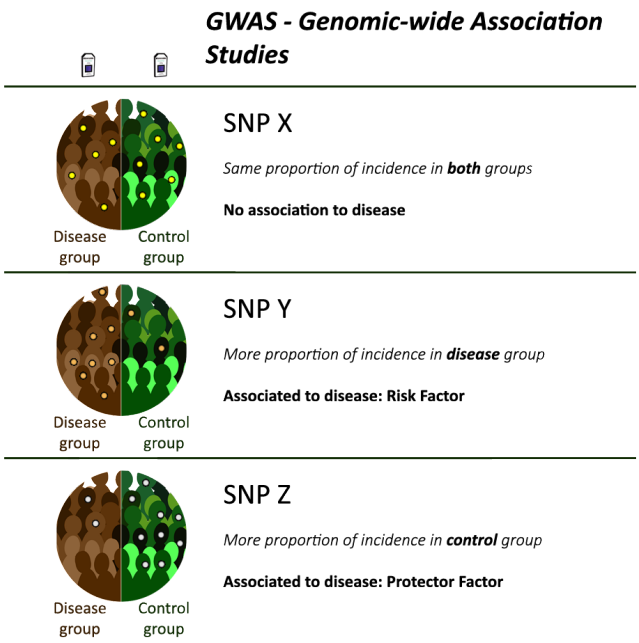
Mutation in tumor-suppressor genes also seem to modify susceptibility to lung cancer. Both *TP53* and *TP63* mutations have been reported in patients with either ADC or SQCC. Interestingly, when a tumor-suppressor gene is mutated, the risk of multiple neoplasms (including lung cancer) becomes increased. For instance, in the Li-Fraumeni Syndrome, a dominant autosomal disorder, more than half of the affected families have inherited mutations in the *TP53* gene and patients present multiple neoplasms in childhood and adolescence. If they survive until adulthood, the risk of tumors, including lung cancer, is highly increased. In turn, *TP63* that encodes p63 (tumor suppressor or transformation-

related protein 63) is also associated with lung cancer, especially in never-smoker females in Asia (26,27)

a. Genome-wide association studies (GWAs)

GWAs are population-based studies used to identify single-nucleotide polymorphisms (SNPs) in different genetic loci. The purpose of these genome-wide investigations is to find genetics alleles that are associated with disease phenotypes. At least 28 SNPs have already been observed to be significantly associated with a risk of NSCLC. Of them, three major loci strongly relate to lung cancer: 15q25 of the genes encoding neuronal nicotinic acetylcholine receptor (nAChR) (subunit genes *CHRNA3* and *CHRNB5*), 5p15 (*TERT* and *CLPTMIL*, genes encoding telomerase reverse transcriptase and cleft lip and palate transmembrane 1-like protein, respectively), and 6p21 (*BAT3* or *HLA-B* associated transcript 3 and *MSH5* or MutS Homolog 5 genes, codifying for large proline-rich protein and a MutS protein involved in DNA repair, respectively). These associations are particularly related to lung cancer in specific ethnic groups, such as Caucasians and Asians (28–30). However, in the vast majority of GWAs, SNPs have demonstrated a strong correlation of polymorphism in two specific genes, those encoding *TERT* and *CLPTM1L*, with lung cancer, indifferently of the ethnic origin of the patients. In particular, *TERT* polymorphisms are especially associated with ADC in never-smokers. Moreover, GWAs strongly suggest that both *TERT* and *CLPTM1L* polymorphisms actually modify the susceptibility to further develop a lung cancer.(31–35)

Figure 2. Histological Types of Lung Cancers.

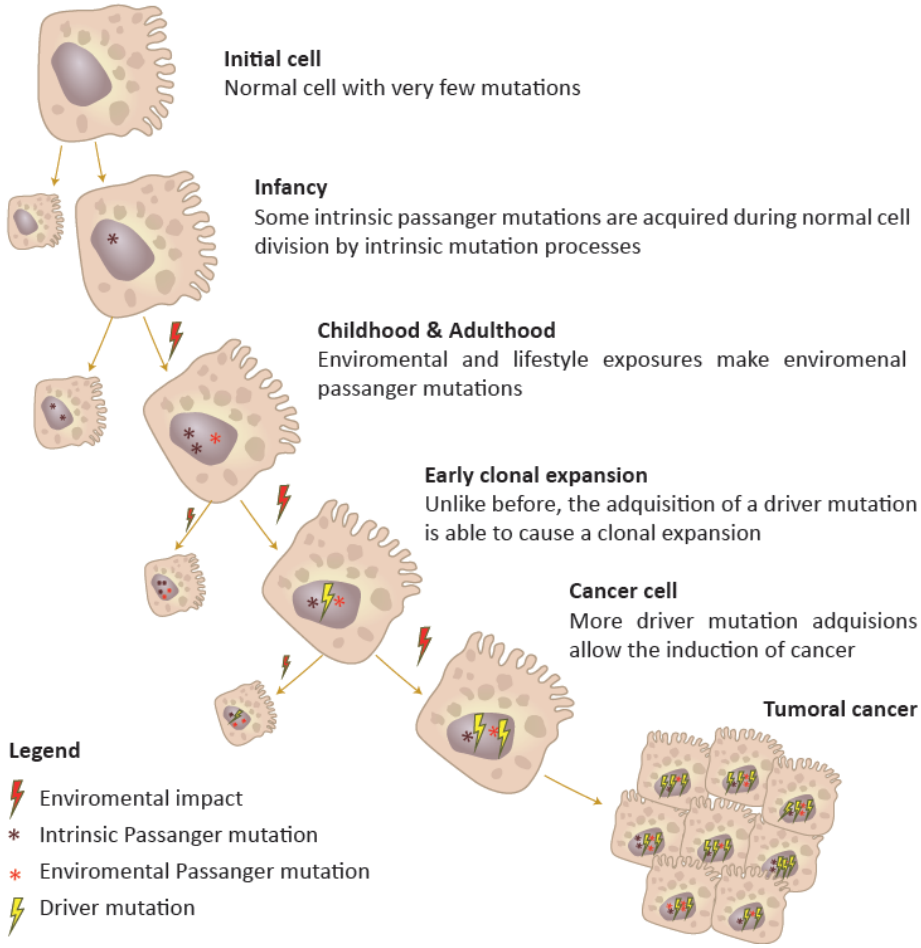


2.3 CARCINOGENESIS AND CANCER HALLMARKS

a. Field Change Cancerization

Field 'cancerization' or 'effect' denotes a large variety of loco-regional changes occurring on the surface of tissues that are exposed to carcinogens for a relatively extended period. These cellular and molecular changes, in otherwise apparently healthy cells, predispose to the occurrence of cancerous lesions. The lung, and especially the bronchial epithelium, is a perfect example of field cancerization. A predisposing genetic background along with long-term exposure to tobacco and/or environmental carcinogens, and an appropriate lung tissue microenvironment result in a field susceptibility that could trigger cancer initiation, evolution and progression (36,37).

Figure 3. Histological Types of Lung Cancers.

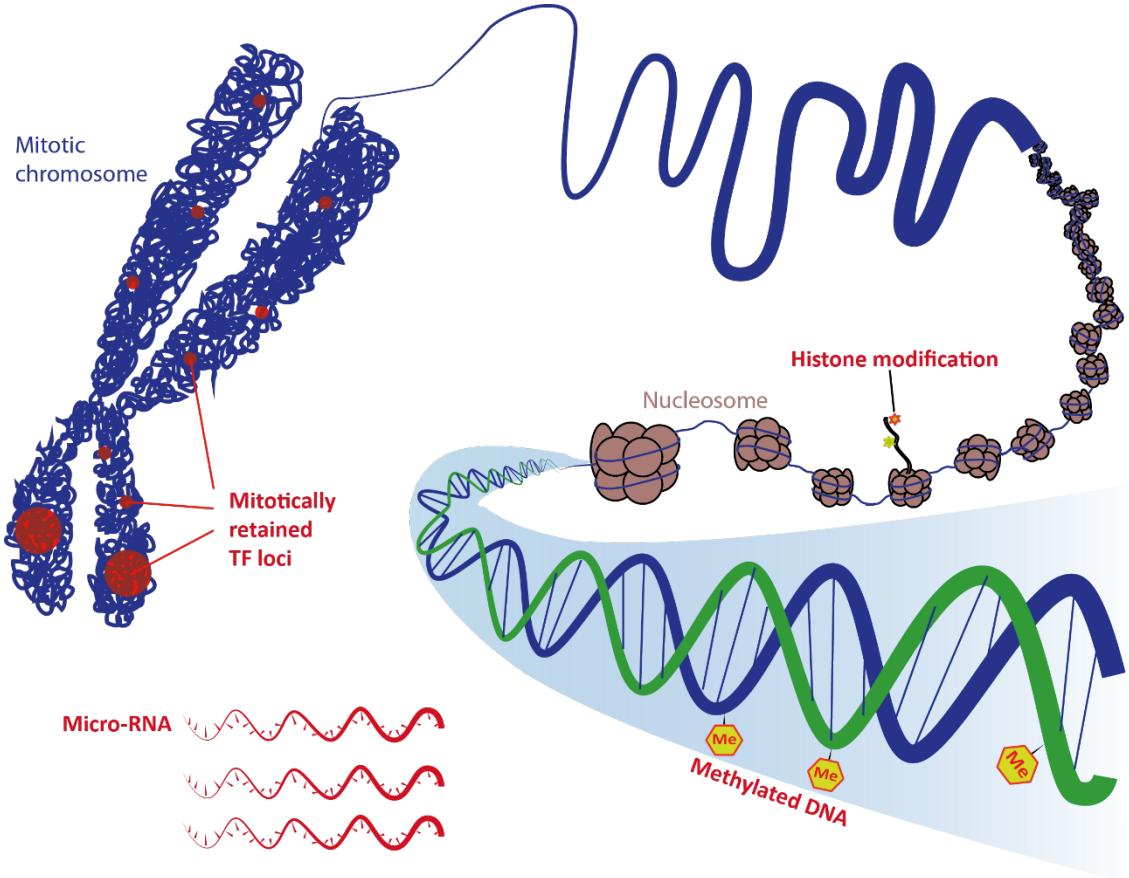


b. Epigenetic Changes

Epigenetic changes are heritable modifications that affect gene expression and other DNA dependent processes without actually changing DNA sequence (38). Although genetic changes play an essential role in ADC tumorigenesis, epigenetic modifications are also

linked to the genesis and progression of cancer, as well as to the response to chemotherapy. These modifications include DNA methylation, and changes in microRNA-mediated regulation and the histone/nucleosome (39). Moreover, different studies have shown a direct association between the presence of methylation of tumor suppression genes and the prognosis of resecable early stage NSCLC. Recently, Daugaard et al., using DNA microarrays, have identified and validated 15 differentially methylated regions (DMRs) in lung ADC, which are absent in the tumor-adjacent normal lung tissue. This study suggests that these DMRs can be used as ADC biomarkers and eventually as targets for novel treatments (40,41).

Figure 4. Histological Types of Lung Cancers.



c. Hallmarks of Cancer

At the beginning of this millennium, Hanahan and Weinberg described the 'Hallmarks of Cancer' as the traits that normal cells slowly acquire in their transformation process to a tumor (42). These authors tried to resume the complexity of this process using a multi-hit model, where different characteristics and discrete genetic alterations progressively add

up until cancer finally develops. Initially, six hallmarks were described, along with two other emerging findings and two more enabling characteristics that facilitate tumor growth and metastatic dissemination (figure 1 and table 1).

Figure 5. Histological Types of Lung Cancers.

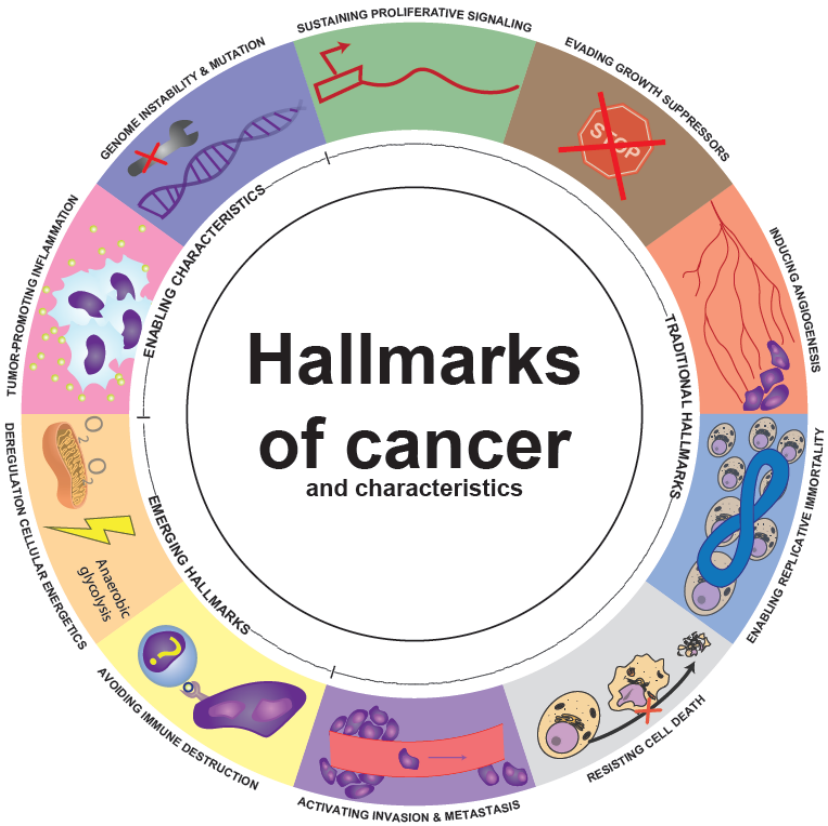


Table 1. Biological hallmarks in lung cancer

Hallmark	Normal Cells	Cancer Cells	Therapeutic Targeting
Sustaining Proliferative Signaling	Cell division starts when intercellular proliferative signals are released (only when needed)	Proliferative signals constantly being used to form rapidly growing tumor structures	EGFR inhibitors
Evading Growth Suppressors	Use growth suppression signals to inhibit unwanted proliferation	Suppressors are repressed and continue to grow out of control	Cyclin-dependent kinase inhibitors
Inducing Angiogenesis	Vascular Endothelial Growth Factor (VEGF) is released to generate new vessels but only if more nutrients are needed	Unlimited growth implicates a high increase on nutrient demands, and VEGF release is increased	Inhibitors of VEGF signaling
Enabling Replicative Immortality	Limited replication is done by progressive and accumulative loss of telomeres in each cell division	Telomerase production allows telomere replication, which in turn results in infinite replication	Telomerase Inhibitors
Resisting Cell Death	Programmed (apoptosis) and necrotic cell death, eliminates cells with a damaged DNA	Apoptosis is attenuated, producing increased cell proliferation, cancer progression and resistance to therapy (43,44)	Pro-Apoptotic BH3 mimetics
Activating Invasion & Metastasis	Organized growth with differential limits.	Tissue barriers are broken and the tumor can invade other organs or vascular and lymphatic vessels (to migrate to other organs)	Inhibitors of HGF/ c-Met
Avoiding Immune Destruction	T-lymphocytes look for surface markers to detect abnormal cells and destroy those with an aberrant behavior	The immune system can be evaded by multiple pathways, mainly avoiding the expression of cell markers	Immunotherapy
Deregulation of Cellular Energetics	Oxygen obtained from blood supply is used to convert glucose to energy	Higher and unreachable nutrient supply is needed. Anaerobic glucose metabolism occurs	Aerobic Glycolysis Inhibitors
Enabling characteristics			
Tumor-promoting Inflammation	Equilibrium between nutrients, inflammatory cells and free radicals is required to produce optimal conditions for normal cell growth and replication	Inflammation modifies cell proliferation, survival, apoptosis and angiogenesis, facilitating the release of reactive oxygen species, promoting carcinogenesis and favoring metastasis (45–48)	Selective anti-inflammatory drugs
Genome Instability & Mutation	Progressive addition of different hallmarks	Gain susceptibility to both genomic alterations and the appearance of driver mutations These genomic changes contribute to the multi-step (or multi-hit) process of carcinogenesis (49,50)	PARP inhibitors

Chapter 3
Genomic Alterations in Lung Adenocarcinoma

3.1 DRIVER MUTATIONS IN LUNG ADENOCARCINOMA

As we have mentioned, this multi-hit and multi-step carcinogenesis model implies that patients with an intrinsic susceptibility (epigenetic modifications or genome heritable traits) exposed to deleterious factors and with an "appropriate" tumoral-peritumoral environment are predisposed to gain specific somatic genetic alterations (see next section) that trigger an initial clonal cell expansion. At the same time, the aforementioned processes continue to add hallmarks and potentiate an abnormal cell proliferation. This dynamic model conceptualizes cancer as an evolutionary process, where a single cell acquires 'advantageous' genomic alterations, allowing itself to proliferate without control, invade and metastasize.

a. Somatic Alterations in Cancer Genome

Genetic alterations are necessary for oncogenesis. Moreover, all malignant cells show DNA modifications at some point during abnormal proliferation. Although these alterations, which are intrinsic to cancer, can be inherited, most of them are the result of errors when DNA becomes copied during cell cycle. In adulthood, DNA has been copied around 30 trillion times, and a cancer-related mutation can occur at any time, with the probability increasing with the passing of years. These acquired changes in DNA are known as 'somatic mutations' or, using a better expression 'somatic genomic alterations' (since not all the DNA modifications are mutations). However, not all these changes are related with the development of cancer. Those somatic genomic alterations that are actually involved in carcinogenesis are known as "driver" alterations, whereas those that are not, are called "passenger" alterations (51,52) (figure 2).

Both pulmonary ADC and squamous cell carcinoma have a high mutational burden compared with other cancers. Interestingly, mutated oncogenes considered as therapeutically targetable predominate in the former. Moreover, when the whole exome of twelve different cancers was sequenced, more than 75% of pulmonary ADC showed driver genomic alterations (53). The frequency of these driver alterations can vary depending on the ethnicity, sex or smoking status, but no differences can be found in different lung ADC stages (54). Table 2 lists the most frequent driver alterations according to TCGA data and the cBioPortal for Cancer Genomics software (open source) (12,55,56).

Table 2. Driver alterations in lung Adenocarcinoma

	Frequency	
	TCGA data	cBioPortal data
Mutations		
KRAS	32.2%	33%
EGFR	11.%	14%
BRAF	7.0%	10%
NF1	8.3%	11%
MET ex14	4.3%	8%
RIT1	2.2%	2%
ERBB2	1.7%	1%
MAP2K1	0.9%	<1%
NRAS, HRAS	0.8%	<1%
Amplifications		
MET	2.2%	4%
ERBB2	0.9%	3%
Translocations		
ROS1	1.7%	2%
ALK	1.3%	3-8%
RET	0.9%	1%

Abbreviations; *EGFR*, Epidermal Growth Factor Receptor gene; *KRAS,* Kirsten Rat Sarcoma viral oncogene homolog; *BRAF*, B-Raf proto-oncogene; *NF1*, Neurofibromin gene; *MET*, MET proto-oncogene receptor tyrosine kinase; *ALK*, Anaplastic lymphoma kinase gene; *ROS1*, C-ros oncogene 1 receptor tyrosine kinase; *RIT1*, Ras-like without CAAX 1; *ERBB2*, erb-b2 receptor tyrosine kinase 2; *MAP2K1*, mitogen-activated protein kinase kinase 1; *RET*, ret proto-oncogene.

b. Epidermal Growth Factor Receptor gene (*EGFR*) mutations

EGFR is one of the most studied oncogenes related to lung ADC, being located on the short arm of chromosome 7. The *EGFR* family encodes proteins that belong to the cell-surface tyrosine kinase receptor family, and consists of four members: *EGFR* (*HER1* or *ErbB1*), *HER2* (*ErbB2*), *HER3* (*ErbB3*) and *HER4* (*ErbB4*) (57–60). These act as transmembrane glycoproteins, and regulate multiple cell processes including apoptosis, cell motility, angiogenesis and proliferative signaling, and also have an impact on carcinogenesis at multiple levels (61,62). *EGFR* is mutated in 10-16% of ADC, with this percentage being much higher in non-smoking women, especially in Asians (where it reaches a frequency of more than 60%) (63–65). Two different somatic alterations account for more than 90% of the total. One is the L858R mutation (substitution of arginine for leucine at codon 858 in exon 21), which represents 45-50% of the cases, and the other is the E746_A750 deletion (in exon 19) that occurs in 45% of the subjects. In the early stages of the disease, ADC with *EGFR* somatic alterations has a better prognosis than the "wild-type" tumor after curative resection. Furthermore, even in advanced ADC the presence of *EGFR* alterations positively changes survival due to the genomic-guided therapy with *EGFR* tyrosine kinase inhibitors (65–69).

c. Kirsten Rat Sarcoma viral oncogene homolog (*KRAS*) mutations

KRAS is one of the three members of the so-called RAS family, along with *HRAS* and *NRAS*. All of them encode low molecular weight proteins that bind to the Guanosine-Triphosphate (GTP), having crucial roles in monitoring the activity of signaling pathways that control normal cell proliferation (70). Moreover, *KRAS* mutations were the first somatic alterations that were identified in lung cancer, and despite being a potential therapeutic target, their significance in the clinical setting still remains controversial (71). Besides, they are also the most common mutations detected in lung ADC (33%), being more frequently detected in older men, smokers, and in large-sized solid tumors and poorly differentiated ADC (72–74). Mutations in codon 12 are the most frequently detected (75% of the total) and result in the substitution of glycine for cytosine (Gly12Cys), valine (Gly12Val) or aspartic acid (Gly12Asp), meanwhile mutations in codon 13 are much less observed (around 7%). Unlike *EGFR* mutations, those occuring in *KRAS* are strongly related with a poorer prognosis in both early stages of ADC and advanced disease. Unfortunately, the attempts to use guided-therapies to target this mutation-phenotype have been extraordinarily frustrating up to now (72,75–78).

d. B-Raf proto-oncogene (*BRAF*) mutations

BRAF encodes a protein called B-Raf that constitutes a crucial step in the RAS-mitogen activated protein kinase (RAS-MAPK) signal pathway. *BRAF* mutations are present in 7-10% of patients with pulmonary ADC, and the vast majority of these mutations are characterized by the substitution of valine by glutamate (Val600Glu or V600E) in exon 15 (79,80). Compared with other lung cancers, *BRAF* mutations are almost exclusive to ADC, although their frequency is low compared with that in other extrathoracic cancers such as melanoma (50-66%) and colorectal carcinoma (>15%). Moreover, this driver mutation is more likely to be observed in smokers and women, and can be targeted by B-Raf protein inhibitors (previously experienced in other cancers). Unlike *EGFR* or *KRAS* alterations, the presence of *BRAF* mutations are not associated with changes in prognosis (70,81,82).

e. Neurofibromin gene (*NF1*) mutations

NF1 is an oncogene encoding the neurofibromin protein. This gene is located in chromosome 17 and is composed by 60 exons, making it one of the largest genes in the human genome. This oncogene has been widely described in the context of type 1 neurofibromatosis, and acts as a tumor suppressor with a negative-regulation of the *RAS* oncogene (83,84). Neurofibromin also regulates cell adhesion, migration and survival, producing a proapoptotic effect. Patients with neurofibromatosis type 1 are considered at high risk of developing malignancies. It should be noted that since TCGA data of somatic mutations are available, *NF1* mutation has become a potential therapeutic target both in

ADC and SQCC. Patients with lung cancer and *NF1* mutation have a concomitant mutation in *KRAS* in 15% of the cases, but in around 70% exhibit no other somatic alteration. It is worth noting that patients with *NF1* alterations in the tumor and those with *KRAS* abnormalities share similar clinical characteristics and prognosis (85).

f. MET proto-oncogene receptor tyrosine kinase (*MET*) amplifications and mutations

MET is an oncogene that encodes for the transmembrane MET tyrosine receptor kinase, with only one known ligand (the hepatocyte growth factor or HGF). The presence of *MET* alterations has a negative impact on prognosis, since amplifications of this gene are related with resistance to *EGFR*-guided therapy in patients with advanced disease, and a high *MET* oncogene copy number is associated with worse prognosis in patients with localized disease. However, *MET* mutations (mutually exclusive with those occurring in *KRAS-EGRF*), despite being identified with a relative high frequency in ADC, have not been related with an oncogenic potential (59,86–89).

g. Anaplastic lymphoma kinase gene (*ALK*) translocations

The *ALK* gene is located on chromosome 2 and encodes a transmembrane tyrosine kinase. Nearly 30 different *ALK* fusions have been described, including the *EML4-ALK* fusion, which is frequently observed in lung ADC (90). This fusion is created by an inversion of the short arm of chromosome 2 that binds exons 1-13 of *EML4* (echinoderm microtubule associated protein like 4) to exons 20-29 of *ALK*, resulting in the synthesis of a chimerical protein with constitutive ALK activity (91–93). Patients with *ALK*-rearranged ADC are usually young, never-smokers and women, showing moderately or poorly differentiated peripheral tumors (94,95). In general, *ALK* alterations are mutually exclusive with *KRAS-EGFR* mutations, having prognosis implications due to the impact of guided-therapies (96).

h. C-ros oncogene 1 receptor tyrosine kinase (*ROS1*) translocations

ROS1 is an oncogene that encodes tyrosine kinase receptor, being phylogenetically related to *ALK*. Unlike *ALK* translocations, *ROS1* rearrangements include one of twelve different partner proteins, and in lung ADC its fusion with *CD74* (cluster of differentiation 74), *EZR* (codifying protein ezrin), *SLC24A2* (encoding the sodium/potassium/calcium exchanger 4) or *FIG* (encoding the fused in glioblastoma protein) genes has emerged as a new driver alteration with promising therapeutic implications. In NSCLC patients the presence of a *ROS1*-rearrangement is specific for ADC, being frequently observed in Asiatic young women and never-smokers (97–99)

3.2 MOLECULAR PROFILING IN LUNG ADC: WHEN AND HOW?

The complete genetic profile of lung ADC is not easily available in standard clinical practice due to the needs of relatively large tissue samples, which often involve the use of invasive techniques, as well as a good molecular biology laboratory, with properly trained personnel, and the elevated costs of the procedure. For these reasons, the realization of strongly directed molecular tests, aimed at the identification of genetic markers with clinical implications is recommended. In this regard, a useful genetic marker should: a) be implicated in the tumorigenesis (such as driver alterations) because the pathway suppression could control tumor proliferation, b) have a high prevalence, to justify the benefit of a costly test, c) have a highly sensitive and specific validated test, and d) have a previously designated oncogenic pathway, with an already available targeted therapy. Although some years ago, a panel of experts from IASLC, ATS and ERS recommended molecular testing only for the *EGFR* mutation in advanced ADC, more recent recommendations also include *EML4-ALK* rearrangement in advanced-stages of lung ADC (either locally advanced or metastatic cancer) (100). However, the latest advances in molecular profiling and guided therapies strongly suggest that the screening should already be extended to at least detection of *ROS1* fusions, *BRAF* mutations and *MET* amplifications or exon 14 alterations, performing a wider genomic profiling in any stage of ADC (4,69,101,102). This will give a more precise scenario of the phenotype epidemiology of this cancer, acting as a strong stimulus for oriented translational research (103).

The first step for the entire process is to identify the origin of the tumor using immunohistochemical techniques in the available sample. Then, the genetic profile is obtained through different techniques such as fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR) or immunohistochemistry. For this, surgical or core-needle samples are preferred due to their larger size. However, molecular techniques can also be applied in smaller samples, such as those obtained in non-invasive or semi-invasive procedures. In this regard, multiple studies have confirmed the utility of even cytological samples obtained by endobronchial ultrasound (EBUS) to perform the molecular study (104–107). However, although a cell block can be obtained by EBUS in most cases (108), there is still controversy on its advantages and disadvantages with respect to the on-site smear in identifying driver alterations (109,110).

Chapter 4
Updated Pathological Classification of Adenocarcinoma

Adenocarcinoma has become the most common histological subtype of lung cancer in most countries. 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 proposal of ADC classification that was finally included unchanged in April 2015 in the 4th edition of the WHO Classification of Tumors of the Lung, Pleura, Thymus and Heart (111). Previous editions based the diagnosis of lung cancer on routine histological criteria obtained from resection samples, but the new classification also integrates immunohistochemistry, and gives specific terminology and diagnostic criteria to smaller biopsies and cytology samples. These criteria would be very helpful for clinicians and patients since around 70% of lung cancers are detected now in advanced stages being unresectable. Moreover, patients would be treated with more personalized chemotherapy and/or radiotherapy with the use of the new criteria. Thus, it is very important to differentiate between ADC and other lung tumors, even in small biopsy specimens. Many tumors show clear morphologic features, but if the sample showed no clear squamous or glandular features, a minimal immunohistochemical workup with specific markers would make the difference. At the moment, TTF-1 (thyroid transcription factor 1) and p40 (which recognizes the ΔNp63-a p63 isoform) are the best markers for ADC and SQCC, respectively (4,8,111–115).

The new ADC classification has interesting innovations. For instance, the term bronchioloalveolar carcinoma (BAC) is no longer used. However, tumors formerly named mucinous BAC are now classified as invasive mucinous ADC, whereas the new name for previously called non-mucinous BAC is lepidic-predominant ADC (111). There is also a new subtype called micropapillary ADC, which has a poorer prognosis. In addition, there are new terms such as AIS (‘in situ’ ADC) and minimally invasive ADC (MIA). Moreover, comprehensive histological subtyping based on the predominant subtype is recommended for invasive lung ADC, and the term “mixed subtype” is not used anymore.

4.1 PREINVASIVE LESIONS

a. Atypical adenomatous hyperplasia

This is a small (usually 0.5 cm or even less) atypical proliferation of type II pneumocytes along preexisting alveolar walls, which resembles but falls short of diagnostic criteria for non-mucinous AIS. Atypical adenomatous hyperplasia is most commonly diagnosed as an incidental histologic finding, which is present in 5-20% of lung cancer resection specimens. The appearance of this atypical proliferation in CT scan is the presence of small ground glass nodules of 5 mm or less (111).

b. In Situ Adenocarcinoma (AIS)

This has been considered as a preinvasive lesion in the new ADC classification since it grows purely with a lepidic pattern without invasion. Most of the cases are non-mucinous, with a proliferation of type II pneumocytes or club cells (formerly denominated 'Clara cells'). More rarely they may be mucinous, with tall columnar goblet cells and abundant mucin in the apical end. The typical image of non-mucinous AIS in the CT scan is to observe small ground glass nodules, whereas the mucinous subtype often has the form of a solid nodule (111). It is worth noting that if AIS is completely resected, the 5-year disease-free survival reaches 100%.

4.2 MINIMALLY INVASIVE ADENOCARCINOMA (MIA)

This concept was introduced to define a relatively benign form of ADC, with nearly a 100% 5-year disease-free survival. MIA refers to a small (≤3 cm) solitary ADC with predominant lepidic growth having an invasion of 5 mm or less. Most of these tumors are non-mucinous, although the mucinous form also exists. Similarly to AIS, while the non-mucinous MIA typically shows ground glass nodes in the CT scan (with a solid component measuring 5 mm or less), the mucinous tumor presents as a solid nodule (111).

4.3 INVASIVE ADENOCARCINOMA

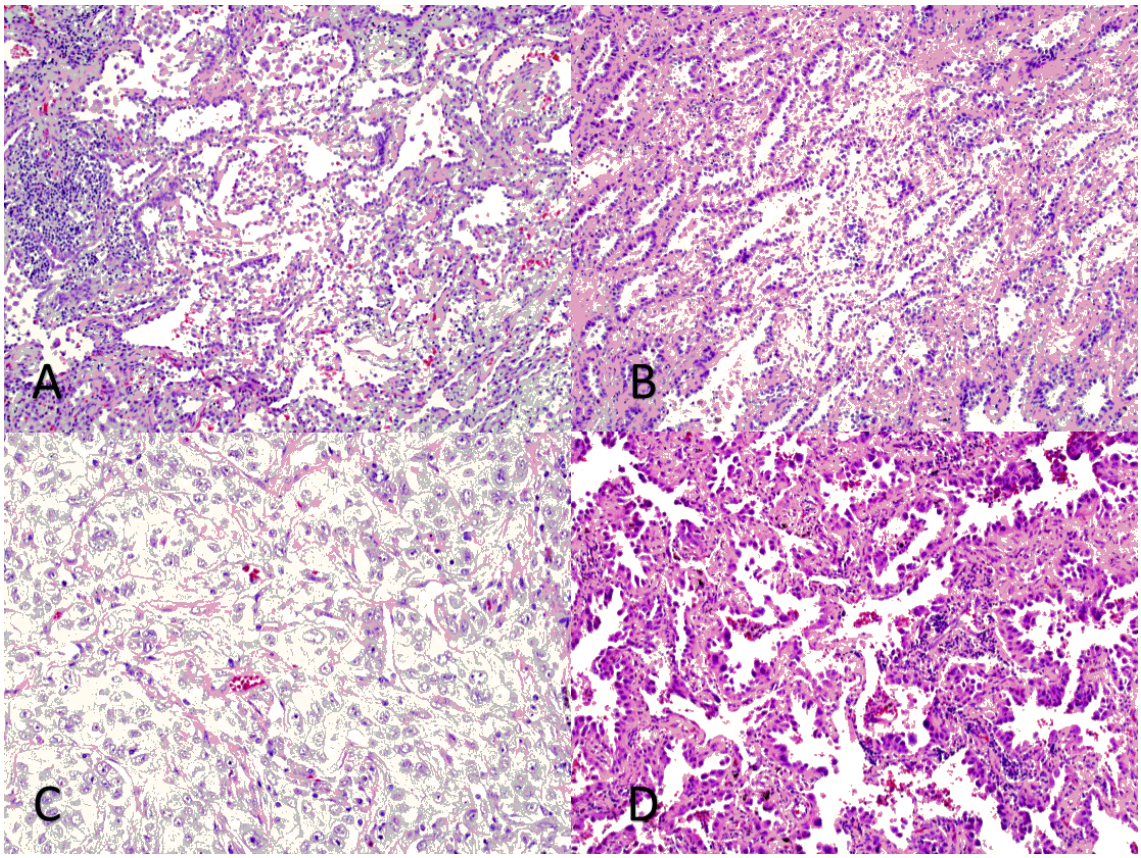
Invasive ADC is classified according to predominant findings. For this, the use of a comprehensive histological subtyping is mandatory, since it allows the estimation of the percentages of the different components. The latter is currently expressed in a semi quantitative fashion, with 5-10% increments. Tumors of mixed characteristics but containing a predominant lepidic growth pattern of type II pneumocytes and/or club cells (formerly known as non-mucinous BAC), which have an invasive component >5 mm are considered as 'lepidic predominant ADC'. Moreover, as previously mentioned a micropapillary predominant subtype has been added to the new classification. The signet ring and club cell carcinoma subtypes are characterized by a relatively high percentage of these features. Although the latter are commonly observed in the solid subtype, they can also show acinar or papillar patterns. Interestingly, there is a good correlation between the amount of the ground glass and the solid component in the CT, and the lepidic growth and the invasion of the tissue, respectively (111).

4.4 ADENOCARCINOMA VARIANTS

The variants of lung ADC accepted today are invasive mucinous, colloid, fetal and enteric ones. The invasive mucinous ADC (formerly known as mucinous BAC) frequently associates *KRAS* mutation and lack of *TTF*-1, and is also characterized by multicentric lung lesions. Histologically, these tumors show different amounts of lepidic, acinar,

papillar or micropapillary growth modalities, all of them characterized by the already mentioned columnar cells with abundant apical mucin and small base-oriented nuclei. In this case, the CT scan frequently shows localized or multifocal consolidation, conforming nodules or lobar involvement, as well as air bronchogram (111).

Figure 6. Histological Types of Lung Cancers.



A. In Situ Adenocarcinoma; B. Minimally invasive Adenocarcinoma; C. Invasive Adenocarcinoma; D. Lepidic Adenocarcinoma.

Chapter 5
Lung Cancer Staging And Therapy

5.1 THE EIGHTH EDITION OF THE TUMOR, NODE AND METASTASIS (TNM) CLASSIFICATION OF LUNG CANCER

In order to understand how the treatment and classification of patients is performed, it is important to explain the basis of the TNM staging classification and to make a schematic review about its story. In 2015, a new TNM proposal was made, being accepted in 2016. The 8th edition of TNM classification is based on the International Association for the Study of Lung Cancer (IASLC) database which finally included 77,156 patients (70,967 with NSCLC and 6,189 with SCLC) between 1999 and 2010. The main changes in relation to the seventh edition were: in the size (T), every centimeter has prognostic implications and divides the tumor into different T groups (from 1cm to 5cms), between 5 and 7 cms are now considered as T3, and tumors greater than 7 are now T4; the distance from endobronchial tumors to carina are not taken into account anymore; the oligometastasis are now considered as M1b; and the size of the part-solid adenocarcinomas is defined by the solid component on CT scan and by the invasion component on pathological analysis (11,116). The treatment of patients with lung cancer is based on the oncological stage that results on the grouping of the different categories of T, N and M, as can be seen in table 3.

Table 3. Oncological Stage based in the 8th edition of TNM classification

STAGE	T	N	M	5-YEARS SURVIVAL
Occult tumor	Tx	N0	M0	
0	Tis	N0	M0	
IA1	T1mi	N0	M0	92%
	T1a	N0	M0	
IA2	T1b	N0	M0	83%
IA3	T1c	N0	M0	77%
IB	T2a	N0	M0	68%
IIA	T2b	N0	M0	60%
IIB	T1a, T1b, T1c	N1	M0	
	T2a, T2b	N1	M0	53%
	T3	N0	M0	
IIIA	T1a, T1b, T1c	N2	M0	
	T2a, T2b	N2	M0	
	T3	N1	M0	36%
	T4	N0	M0	
	T4	N1	M0	
IIIB	T1a, T1b, T1c	N3	M0	
	T2a, T2b	N3	M0	26%
	T3	N2	M0	
	T4	N2	M0	
IIIC	T3	N3	M0	13%
	T4	N3	M0	
IVA	T1, T2, T3, T4	N1, N2, N3	M1a	10%
	T1, T2, T3, T4	N1, N2, N3	M1b	
IVB	T1, T2, T3, T4	N1, N2, N3	M1c	<1%

5.2 TREATMENT

The treatment of lung cancer varies depending on clinical/pathological stage. In inoperable lung cancer, the most important predictors of prognosis are the performance scales (Karnofsky scale), TNM stage, weight loss and age (117). The 5-year survival is <15%, clearly conditioned by the extent of the disease at diagnosis (see Table 3). The ACCP recommend a treatment guided by performance status plus TNM stage and decided by a multidisciplinary team (118–120).

Table 4. Treatment by Stage in NSCLC (118–120)

Stage	5-years Survival	First Line	Adjuvant Chemotherapy	Second Line	Other
IA1	92%	Surgery	NO	Radiotherapy	
IA2	83%	Surgery	NO	Radiotherapy	
IA3	77%	Surgery	NO	Radiotherapy	
IB	68%	Surgery	NO	Radiotherapy	
IIA	60%	Surgery	SI	Radiotherapy	
IIB	53%	Surgery	SI	Radiotherapy	
IIB Pancoast	53%	Surgery + Neadjuvant Chemoradiotherapy	NO	Chemoradiotherapy	
IIIA	36%	Induction Chemotherapy + Surgery	SI/NO	Chemoradiotherapy	
IIIB	26%	Chemoradiotherapy	-	Chemotherapy alone or Radiotherapy alone	
IIIC	13%	Chemoradiotherapy	-	Chemotherapy alone or Radiotherapy alone	
IVA	10%	Chemotherapy or Guided Therapy or Immunotherapy	-	Chemotherapy or Guided Therapy or Immunotherapy	Palliative Radiotherapy
IVB	<1%	Chemotherapy or Guided Therapy or Immunotherapy*	-	Chemotherapy or Guided Therapy or Immunotherapy	Palliative Radiotherapy

*The first line treatment of stage IV lung cancer is changing constantly depending on new evidence and driver mutation/PD-1/PDL-1 status. See sections 5.3 and 5.4

5.3 GENOME-GUIDED THERAPY

In November 2004, the first genome targeted therapy was approved by the FDA for the treatment of NSCLC with *EGFR* mutations. Since then, the prognosis of selected patients with advanced ADC and driver mutations has improved substantially. In fact, molecular testing is performed routinely in locally advanced or metastatic ADC since targeted

therapies have been approved and their impact on multiple outcomes has been demonstrated. This is the case of patients with *EGFR* mutations, *EML4-ALK* rearrangement or *ROS1* fusions (69). For instance, ertenolib, gefitinib and afatinib are used in the treatment of locally advanced or metastatic tumors with *EGFR* exon 19 deletion or exon 21 mutations, while osimertinib, olmutinib and osimertinib are employed in the case of *EGFR* T790M mutations (121–127). Crizotinib, ceritinib and alectinib in turn are used in similar tumors, which in this case show *ALK* alterations. If a *ROS1* translocation is present, crizotinib can be used to treat the patients (98,128–132). More recently, promising evidence has been published with the use of crizotinib in tumors with *MET* exon 14 alterations or amplification, and dabrafenib plus trametinib in patients with *BRAF* mutations (102,133). Table 3 summarizes the approved genome-guided therapies for lung ADC and their present indications.

5.4 IMMUNOTHERAPY

Immunotherapy is a relatively novel approach for cancer treatment, being based on the stimulation of the patient's immune system to induce a cellular-humoral response that attacks and destroys the malignant cells. Immunotherapy can be active or passive, with both being either specific or non-specific. The active immunotherapy consists in the activation of the host's immune system to induce a specific response, whereas passive immunotherapy is based on the administration of antibodies that will directly kill cancer cells, without interacting with the patient´s immune system. Therapy is specific if it results in a particular immune response or as general if it involves a wider immunological reaction (134).

Many scientific advances in cancer treatment are being developed in the field of active immunotherapies, whose main modalities are therapeutic vaccines and checkpoint inhibitors (135,136). The former stimulates the host immune system to generate a prolonged immunological response by recognizing tumor antigens. The vaccines can be antigen-specific or addressed to the whole-tumor, and have already been studied in the adjuvant setting, as first line and maintenance treatments, but unfortunately no positive results have been found up to now. (137–139). Immune checkpoints, in turn, are inhibitory trails that control the duration and intensity of the immune response to reduce the damage in normal tissues. There are two targetable checkpoints that have been widely studied in the last years: the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and the programmed death-ligand 1/ programmed cell death protein 1 (PD-L1/PD-1) pathway (140).

Table 4. Main genome-guided therapies employed with ADC and their indications

	Approved	FDA indication	EMA indication
Erlotinib (Tarceva©)	FDA: Nov. 2004 EMA: Sep. 2005	First-line in metastatic NSCLC with *EGFR* exon 19 deletions or exon 21 (L858R) mutations	Treatment of locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen or switch maintenance treatment in stable disease
Gefitinib (Iressa©)	FDA: Jul. 2015 EMA: Jun. 2009	First-line in metastatic NSCLC with *EGFR* exon 19 deletions or exon 21 L858R mutations	Treatment of locally advanced or metastatic NSCLC with activating *EGFR* mutations
Crizotinib (Xalkori©)	FDA: Aug. 2011 EMA: Oct. 2012	Treatment of locally advanced or metastatic *ALK* positive NSCLC detected by a FDA-approved test	First-line and therapy of previously treated advanced *ALK* positive NSCLC
Afatinib (Giotrif©)	FDA: Jul. 2013 EMA: Sep. 2013	First-line in metastatic NSCLC with *EGFR* exon 19 deletions or exon 21 (L858R) mutations detected by a FDA-approved test Second-line in advanced SQCC with disease progression after treatment with platinum-based chemotherapy	Treatment of locally advanced or metastatic NSCLC with activating *EGFR* mutations Treatment of locally advanced or metastatic NSCLC of squamous cancer progressing on or after platinum-based chemotherapy
Ceritinib (Zykadia©)	FDA: Apr. 2014 EMA: May 2015	Treatment of metastatic *ALK* positive NSCLC with disease progression on or that are intolerant to crizotinib	Treatment of locally advanced or metastatic *ALK* positive NSCLC
Osimertinib (Tagrisso©)	FDA: Nov. 2015 EMA: Feb. 2016	Treatment of locally advanced or metastatic NSCLC with *EGFR* T790M mutations as detected by an FDA-approved test, that has progressed on or after *EGFR* tyrosine kinase inhibitor therapy	Treatment of locally advanced or metastatic NSCLC with *EGFR* T790M mutations.
Alectinib (Alecensa©)	FDA: Dec. 2015	Treatment of *ALK*-positive metastatic NSCLC who has progressed on or is intolerant to crizotinib	
Crizotinib (Xalkori©)	FDA: Mar. 2016 EMA: Jul. 2016	Treatment of metastatic *ROS1*-positive NSCLC	Treatment of advanced *ROS1*-positive NSCLC
Olmutinib (Olita©)	FDA: granted BTD	Treatment of locally advanced or metastatic *EGFR* T790M mutation in NSCLC	
Dabrafenib (Mekinist®) +Trametinib (Tafinlar®)	FDA: granted BTD	Treatment of metastatic *BRAF* V600E-positive and previously treated NSCLC	
Osimertinib	FDA: granted BTD	Treatment of metastatic NSCLC with *EGFR* T790M mutations and TKI resistant disease	

Abbreviations: FDA, U.S. Food and Drug Administration; EMA: European Medicines Agency; FDA BTD: granted breakthrough therapy designation.

Data obtained from online database of FDA (www.fda.org) and EMA (www.ema.europa.eu/ema/)

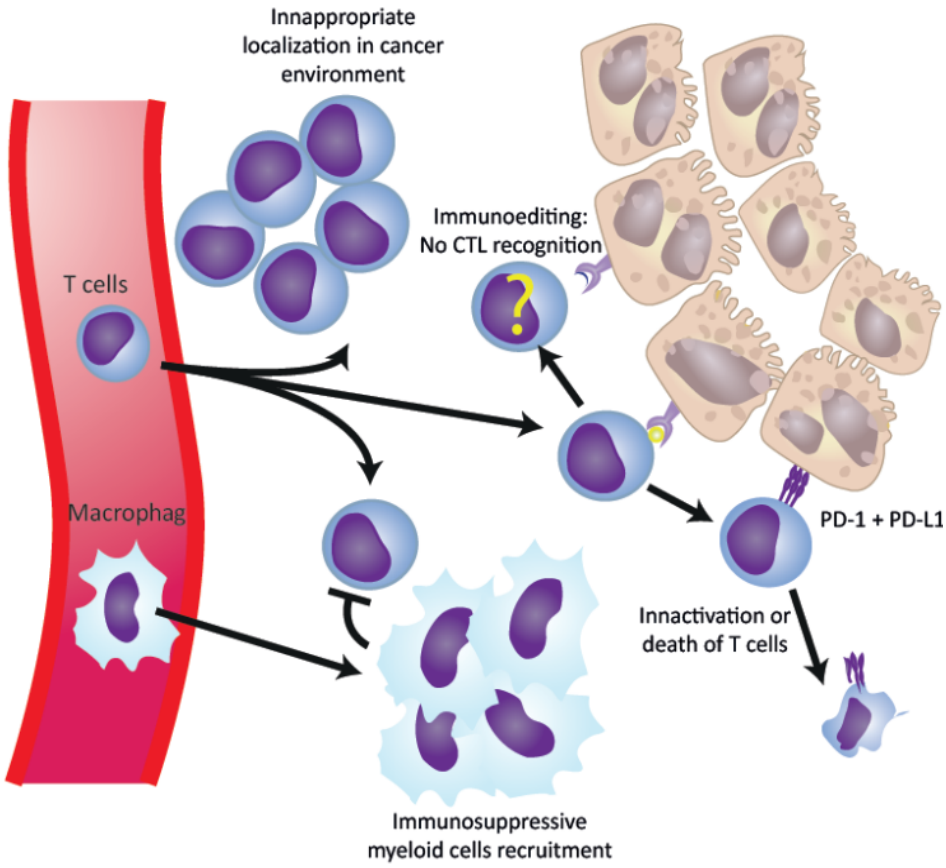
a. CTLA-4 inhibitors

Two humanized monoclonal antibodies inhibiting CTLA-4 have been tested in clinical trials on patients with NSCLC cancer. In this respect, a trial using tremelimumab in advanced-stage NSCLC showed a good tolerability profile but unfortunately showed no differences in the progression-free survival when used as a second-line agent if compared with the best supportive care (141). Two other clinical trials (ClinicalTrials.gov, numbers NCT02000947 – NCT02352948), that are now in the recruitment phase, have been designed to compare dual checkpoint inhibition (anti PD-L1 and CTLA-4) using tremelimumab and durvalumab with the standard therapy (142,143).

b. PD-1/PD-LI inhibitors

Under normal conditions, the PD-1 protein checkpoint protects against inflammation and autoimmunity. When a neoplasm occurs, PD-1 binds to the PD1-L1 and causes immunosuppression, preventing the immune system from attacking the tumoral cells (144). To date, FDA has approved three PD-1/PD-L1 inhibitor drugs for the treatment of advanced-stages of NSCLC. These are nivolumab (Opdivov©, October 2015), pembrolizumab and aterolizumab (Keytruda© and Tecentriq©, respectively, both in October 2016). Nivolumab is an IgG4 monoclonal antibody that blocks PD-1 receptors expressed on activated T cells. Multiple clinical trials (CheckMate trials) have evaluated nivolumab versus docetaxel in advanced-stage NSCLC, showing an overall improved survival and a significantly better progression-free survival in the nivolumab group, with an acceptable tolerability and toxicity profile, turning this treatment into the second-line gold standard therapy in such cases (145,146). Pembrolizumab, previously called lambrolizumab, is a humanized IgG4 immunoglobulin with a high affinity for PD-1. Many clinical trials (KEYNOTE trials) have shown benefits in the overall response rate (ORR), and the overall survival in a large number of patients with advanced-stage NSCLC when compared with standard therapies, again with an excellent security profile (147,148). Ongoing studies are trying to define if pembrolizumab can be used as a first-line treatment in advanced NSCLC. Finally, a randomized, phase 3 clinical trial (OAK study), with more than a thousand patients from 31 different countries, has shown a better overall survival in patients with a previously treated NSCLC with atezolizumab when compared to docetaxel, irrespectively of PD-L1 expression (149).

Figure 7. Immune response in Lung Adenocarcinoma.



In conclusion, the use of genomic phenotyping of ADC, possible now even in relatively small samples, facilitates a better tumor classification, and allows for a more targeted treatment. For this, two different strategies have been developed, genome-guided therapies, mainly based on blocking the aberrant resultant pathway, and immunotherapy, which can either be active (stimulation of the patient's immune system to produce a specific response) or passive (administration of external antibodies). Although the immune strategy is still being developed, its current results are very promising.

Chapter 6
Hypothesis

The physiopathology of carcinogenesis is a complex process that includes multiple steps. Intrinsic susceptibility, epigenetic changes, exposure to extrinsic deleterious factors, tumoral-peritumoral environment and genotypic changes, produce a misbalance between proliferation and cell death. The somatic genetic alterations called “driver mutations” are considered to be the last step in this process and necessarily trigger the clonal cell expansion. We hypothesized that the driver mutations can be present in the absence of tumor in apparently healthy cells, at least histologically. It is likely that as the lung epithelium is large and its cells share the same environmental exposure, carcinogenic factors can promote loco-regional modifications not only in the future tumor but throughout the exposed tissues and more than one cell can gain molecular alterations simultaneously.

Taking into account the high rate of recurrence in patients with lung neoplasm independent of the clinical stage in which it is detected and the field change cancerization theory, we also believed that the presence of molecular alterations in non-tumoral cells can have prognosis implication and be an unknown pathological mechanism in tumor growth and spread.

Finally, we also hypothesized that in the future, the molecular study of some types of lung cancer can be performed with less invasive techniques since not only the cancer cells can develop genetic changes and molecular techniques can also be performed in cytological or blood specimens.

Chapter 7
Specific Aims

7. SPECIFIC AIMS

The specific aim of this thesis is to demonstrate the presence of cancer-related genomic alterations in non tumoral cells of patients with pulmonary adenocarcinoma, to demonstrate that the presence of this mutations implicate an overall worst prognosis, and to evaluate less invasive techniques for obtaining biological samples and performing molecular genomic-studies.

7.1. Fist paper

The specific aim of this paper was to identify whether the most prevalent driver mutations observed in lung adenocarcinoma were also present in the histologically non-tumoral lung tissue of the same patient and, if this was the case, to assess their potential usefulness as markers of prognosis. The objective was to include patients with localized adenocarcinoma and *EGFR* or *KRAS* mutations who underwent curative resection and obtain normal lung parenchyma samples to extract DNA and perform molecular genomic testing to detect the same driver mutation previously identified in the tumor.

7.2. Second paper

The specific aim of this paper was to evaluate the diagnostic yield of bronchial brushing cytology of peripheral pulmonary adenocarcinoma guided by fluoroscopy plus radial endobronchial ultrasound (radial-probe EBUS) in the detection of driver mutations when compared with histological specimen. This prospective study was designed to evaluate the utility of *Roswell Park Memorial Institute* medium (RPMI) in the preservation of tumoral cells and DNA, and to assess the correlation between the molecular analysis of the brushing specimen and the genomic molecular alterations found in the lung tumor.

7.3. Third paper

The specific aim of this paper was to demonstrate the presence of *EGFR* and *KRAS* mutations in non-tumoral lung cells in patients with localized adenocarcinoma with negative genomic testing in the tumor. This study pretended to confirm the hypothesis that cancer-related mutations can appear in non-cancerous cells even in the absence of molecular alterations in the primary tumor. The objective was to recruit a prospective cohort of patients with pulmonary adenocarcinoma who underwent curative resection and wild type *EGFR* and *KRAS* status in the tumor, and perform molecular testing to *EGFR* and *KRAS* mutations in histologically normal lung samples.

7.4. Additional thesis material: physiopathology Review

The specific aim of this complementary paper was to make and publish in a peer-review journal an exhaustive review in the state-of-the-art of the complex physiopathology of adenocarcinoma carcinogenesis and to describe the pathological and clinical implications that the presence of driver mutations in the tumor have on patients with lung cancer.

7.5. Additional thesis material: Editorial

The specific aim of this complementary paper was to make an editorial discussing the results published recently in the *American Journal of Respiratory and Critical Care Medicine* by Mark et al (150). The aim of this specific article was to elucidate how the immune checkpoints and T-cell immunity could be interrelated in patients with COPD and non-small cell lung cancer.

Chapter 8
Publications

8.1 MANUSCRIPT I

Chalela R, Bellosillo B, Curull V, Longarón R, Pascual-Guardia S, Badenes-Bonet D, et al. EGFR and KRAS Mutations in the Non-Tumoral Lung. Prognosis in Patients with Adenocarcinoma. J Clin Med. 2019 Apr 17;8(4):529. DOI: 10.3390/jcm8040529

8.2 MANUSCRIPT II

Sánchez-Font A, Chalela R, Martín-Ontiyuelo C, Albero-González R, Dalmases A, Longarón R, et al. Molecular analysis of peripheral lung adenocarcinoma in brush cytology obtained by EBUS plus fluoroscopy-guided bronchoscopy. Cancer Cytopathol. 2018 Oct 6;126(10):860–71. DOI: 10.1002/cncy.22053

8.3 MANUSCRIPT III

CANCER-RELATED MUTATIONS IN LUNG PARENCHYMA: AN ADDITIONAL STEP IN FIELD CANCERIZATION HYPOTHESIS? BRIEF REPORT.

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Take-Home Message: *EGFR* or *KRAS* mutations can also be present in histologically normal lung tissue regardless of tumor status.

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ABSTRACT

The acquisition of driver mutations in non-tumoral cells are very important during the carcinogenesis of ADC. Recent studies suggest that cancer-related mutations may not necessarily be present only in malignant cells but also in histologically healthy cells **Objective:** to demonstrate the presence of *EGFR* or *KRAS* mutations in non-tumoral lung cells in patients with localized adenocarcinoma with negative genomic testing in the tumor. **Results:** five mutations in *EGFR* or *KRAS* oncogenes were detected among three patients (9.7%) in the normal lung parenchyma. The exon 21 substitution L858R in *EGFR* was detected in two cases while the exon 19 deletion E746-A750 in the *EGFR*, the codon 12 substitution Gly12Cys (G12C) and Gly12Asp (G12D) in the *KRAS* were detected once. One patient presented three different ones in the normal lung parenchyma (*EGFR*_L858R, *KRAS*_G12C and *KRAS*_G12D). The negative-mutation status of the tumor and the mutations detected in the normal lung parenchyma were confirmed using highly sensitive and specific TaqMan PCR (CAST-PCR). No differences were found in terms of progression (locally or at a distance), progression-free survival or overall survival between both groups during the 18 months follow-up. **Conclusions:** These results confirm the presence of driver mutations in the normal lung parenchyma cells in the absence of mutations in the primary tumour.

Keywords

Adenocarcinoma – Mutations – EGFR – KRAS – Prognosis

INTRODUCTION

Lung cancer, specifically lung adenocarcinoma (ADC), is frequently diagnosed in advanced stage with a global 5-year survival not exceeding 17% (1–3). Even when it's detected at an early-stage, the prognosis of patients with ADC is poor, especially in terms of tumor recurrence (4,5). Pulmonary ADC has an extraordinary high mutational burden and somatic genomic alterations can be found in more than 75% of the cases with a vast proportion of oncogenic driver alterations affected that have potential therapeutic implications (6). These acquisitions of driver mutations in non-tumoral cells are very important during the carcinogenesis of ADC and will necessarily produce a clonal cell expansion, at least taking into account its current definition (7). In the last months two studies have demonstrated the presence of cancer-related mutations in non-tumoral cells of patients with endometriosis and arteriovenous malformations of the brain (8,9). These findings suggest that cancer-related mutations may not necessarily be present only in malignant cells, but also in histologically benign cells. Our group has recently demonstrated that patients with localized lung ADC with *EGFR* or *KRAS* alterations, presented the same driver mutation in non-tumoral lung cells in 21.3% of the cases. These findings were associated with a significantly lower disease-free survival at 12 months (10). Our hypothesis is that cancer-related mutations can appear in non-cancerous cells even in the absence of molecular alterations in the primary tumor during the field cancerization process. Accordingly, the aim of the present study was to demonstrate the presence of *EGFR* or *KRAS* mutations in non-tumoral lung cells in patients with localized adenocarcinoma with negative genomic testing in the tumor.

METHODS

Patients

Patients with early-stage lung ADC with negative mutational status and candidates for curative resection were prospectively recruited in our center, a tertiary teaching-hospital. Tumor and normal lung parenchyma samples were obtained and processed. Thirty-five patients with *EGFR* mutation-negative and *KRAS* mutation-negative lung adenocarcinoma were included. The normal lung parenchyma (NLP) sample was defined as a histologically normal tissue with complete absence of micro-tumor invasion assessed by two expert lung pathologists and obtained in the area of the lung furthest from the tumor (at least 2 cm away from the tumor). Finally, viable non-tumoral DNA was obtained in 31 of these patients and a competitive allele-specific TaqMan PCR was performed to identify the presence of *EGFR* or *KRAS* mutations. The cohort was followed-up during 18 months and clinical data was collected for months 1, 2, 6, 12 and 18. The study was designed and carried out in accordance with the ethical guidelines of the Declaration of Helsinki and European legislation, and the procedure was approved by our Ethics Committee. Informed consent was obtained from all individuals.

Tumor DNA extraction and sequencing.

DNA was extracted from tumoral sections of each sample with the commercially available QIAamp DNA Mini kit (Qiagen, Hilden, Germany). The *EGFR* mutational status was analysed by real-time PCR using the TheraScreen *EGFR* RGQ PCR kit (Qiagen), a highly sensitive assay based on Scorpions® real-time PCR technology and mutation specific ARMS® primers that detect 29 different somatic mutations in the gene. In addition, 18, 19, 20 and 21 exons of the *EGFR* gene, as well as exon 2 of the *KRAS* gene, were analysed in all cases by Sanger sequencing, using BigDye v3.1 (Applied Biosystems, Foster City, CA), being assessed on the 3500DX Genetic Analyzer (Applied Biosystems).

Normal lung parenchyma DNA extraction and sequencing.

DNA was extracted from two sections of 15 μm using the QIAamp DNA Mini kit (Qiagen). Mutational analysis was performed in this case using competitive allele-specific TaqMan PCR (CAST-PCR, Applied Biosystems, 4465804). The following individual assays were used: *EGFR* exon 19 deletions - Hs00000228_mu; *EGFR* p.L858R- Hs00000102_mu; *EGFR* p.T790M - Hs00000106_mu; G719A- Hs00000104_mu; *KRAS* p.G12C- Hs00000113_mu; *KRAS* p.G12V– Hs00000119_mu; *KRAS* p.G12D- Hs00000121_mu; *KRAS* p.G12A - Hs00000123_mu; *KRAS* p.G12R– Hs00000117_mu; and *KRAS* p.G13C- Hs00000125_mu.

Statistical analysis

While categorical variables are described as frequencies and percentages, continuous variables are expressed as mean ± standard deviation. Pearson's Chi-Square or Fisher exact tests were used as appropriate to compare categorical variables among groups. The non-parametric Mann-Whitney U test was used to assess differences between groups. A Log-rank test was used to compare the survival distributions of the two groups. P values ≤ 0.05 were considered statistically significant. Analyses were performed with SPSS 21.0.

RESULTS

The main clinical, functional and tumor characteristics of the cohort are shown in table 1. All patients were stratified in stages based on the TNM classification (IASLC, 8th edition) and only stage I or II patients were included (3). The surgical procedures were performed in accordance with the institution clinical-practice recommendations. The most common procedure was a lobectomy (67.7%) followed by segmentectomy (22.6% and bilobectomy (9.7%). Almost all the patients (30 of 31, 96.8%) were smokers or former smokers.

TABLE 1. Baseline characteristics and comparison between mutated NLP and non-mutated NLP

	Total n = 31	Mutated NLP n = 3	Non-mutated NLP n = 28	p value
Age, mean (SD), yrs.	64.2 (7.2)	60 (6)	64.5 (7.1)	0.29
Current or former smoker, n (%)	**30 (96.8)**	**2 (66.7)**	**28 (100)**	**0.00**
Smoking index, mean (SD), pack-year	53.2 (23)	40 (34.6)	54.6 (22)	0.30
Sex, n (%)				
Male	25 (80.6)	**1 (33)**	**24 (85.7)**	**0.02**
Female	6 (19.4)	**2 (66.7)**	**4 (14.3)**	
Comorbidities, n (%)				
Previous cancer	12 (38.7)	1 (33.3)	11 (39.3)	0.84
Dyslipidemia	10 (32.3)	1 (33.3)	9 (32.1)	0.96
Hypertension	10 (32.3)	0 (0)	10 (35.7)	0.20
Diabetes mellitus	7 (22.6)	1 (33.3)	6 (21.4)	0.63
Alcoholism	9 (29)	0 (0)	9 (32.1)	0.24
COPD	8 (25.8)	0 (0)	8 (28.6)	0.28
Diabetes mellitus	7 (22.6)	1 (33.3)	6 (21.4)	0.63
Ischemic cardiomyopathy	2 (6.5)	0 (0)	2 (7.1	0.63
Chronic kidney disease	1 (3.2)	0 (00)	1 (3.6)	0.73
Lung function tests, mean (SD)				
FEV_1, *% ref.*	74.5 (16.1)	81 (31.1)	74.2 (15.3)	0.56
FVC, *% ref.*	86.9 (16.6)	85.5 (28.9)	86.5 (16.1)	0.93
TLC, *% ref.*	99.9 (13)	77	99.8 (13)	0.09
RV/TLC, *%*	46.2 (11)	38	46.2 (11)	0.47
DL_{CO}, *% ref.*	65.3 (18.8)	79.5 (47)	64.4 (16.4)	0.27
Karnofsky Scale, mean (SD)	93.3 (6)	100	92.8 (6)	0.09

Tumor characteristics				
SUV by PET, mean (SD), cm	6.5 (4.7)	3.4 (2.5)	6.8 (4.7)	0.33
T (tumor size), mean (SD), cm	2.8 (18.4)	1.4 (0.1)	2.9 (1.8)	0.15
N (nodal infiltration), n (%)	3 (9.7)	1 (33.3)	2 (7.1)	0.14
M (metastasis), n (%)	0 (0)	0 (0)	0 (0)	--
Post-operative Stage Groups, n (%)				
I	23 (74.2)	21 (75)	2 (66.7)	0.75
II	8 (25.8)	1 (33.3)	7 (25)	0.75
III - IV	0 (0)	0 (0)	0 (0)	--
Diagnostic tests, n (%)				
PET-CT scan	27 (87.1)	2 (66.7)	25 (89.2)	0.77
Endobronchial Ultrasound (EBUS)	17 (54.8)	2 (66.7)	15 (536)	0.76

Abbreviations: NLP, normal lung parenchyma; SD, standard deviation; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; TLC, total lung capacity; RV, residual volume; DLco, transfer coefficient for CO; SUV, standardized uptake value; PET, positron emission tomography.

We identified five mutations in *EGFR* or *KRAS* oncogenes among three patients (9.7%) in the normal lung parenchyma. The exon 21 substitution L858R in *EGFR* was detected in two cases while the exon 19 deletion E746-A750 in the *EGFR*, the codon 12 substitution Gly12Cys (G12C) and Gly12Asp (G12D) in the *KRAS* were detected once. Surprisingly, in one patient, three different mutations were identified in NLP (*EGFR*_L858R, *KRAS*_G12C and *KRAS*_G12D). More details of the three patients with mutated NLP can be found in Table 2.

TABLE 2. Detailed mutation characteristics and progression.

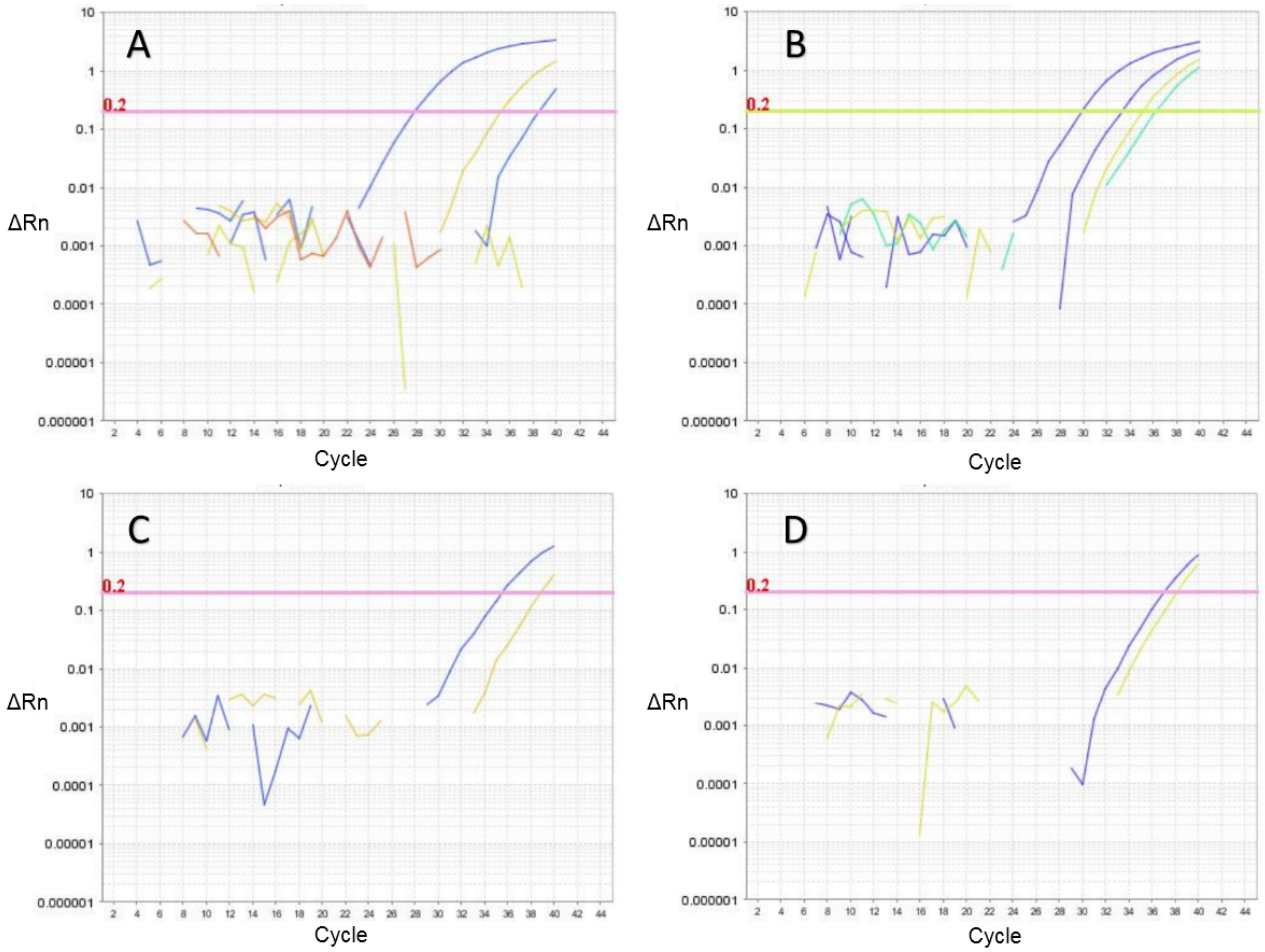
Nº	Age (years)	Sex	TNM	Mutational status in NLP	Distant - local progression	Site of progression
1	69	0	T2AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
2	67	0	T3N1M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
3	56	0	T2AN0M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Adrenal
4	55	0	T1BN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
5	62	0	T2AN1M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Lymph Nodes Local progression
6	58	0	T1BN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
7	59	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Brain Adrenal
8	67	0	T3N0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
9	55	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
10	67	0	T1BN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
11	55	0	T3N0M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Brain Local progression
12	69	0	T2AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
13	78	0	T2AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
14	75	0	T1BN0M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Lymph Nodes Bones
15	73	0	T3N0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
16	66	0	T1BN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
17	53	1	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
18	60	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
19	60	1	T2AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
20	59	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
21	**53**	**0**	**T1AN0M0**	***EGFR* deletion E746-A750** ***KRAS* Wild-type**	**NO**	
22	**64**	**1**	**T1AN0M0**	***KRAS* Gly12Cys** ***KRAS* Gly12Asp**	**YES**	**Liver**

				***EGFR* substitution L858R**		
23	63	1	T1BN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
24	72	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
25	68	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
26	61	1	T1BN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
27	70	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	NO	
28	67	0	T1AN0M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Lymph Nodes
29	80	0	T2AN0M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Adrenal
30	65	0	T2AN0M0	*KRAS* Wild-type *EGFR* Wild-type	YES	Brain
31	**63**	**1**	**T1AN0M0**	***EGFR* substitution L858R** ***KRAS* Wild-type**	**NO**	

Abbreviations: EGFR, Epidermal Growth Factor Receptor gene; *KRAS*, Kirsten Rat Sarcoma viral oncogene homolog.

In these three patients, the negative-mutation status of the tumor was confirmed for the specific mutation detected in the NLP using highly sensitive and specific TaqMan PCR (CAST-PCR). The confirmation of the positive-mutation status in the NLP was also performed for the five specific mutations previously mentioned. In all the assays, the PCR efficiency was between 95 and 105%. To improve specificity and avoid false positives we only considered assays for *EGFR* mutations as positives when the amplification occurred before the cycle 35. For the two *KRAS* mutations detected, the amplification occurred between the cycle 35 and 38, however in both cases the mutation was confirmed. The amplification plots are shown in Figure 1.

FIGURE 1. Amplification plots of detected mutations.



A. Amplification plot for *EGFR* exon 19 deletion E746-A750. B. Amplification plot for *EGFR* exon 21 substitution L858R C. *KRAS*, codon 12 substitution Gly12Cys. D. *KRAS*, codon 12 substitution Gly12Asp.

Mutated NLP vs non-mutated NLP: clinical outcomes, recurrence and survival

We only found differences between both groups in terms of tobacco status and gender. Patients in the mutated NLP group were significantly less-frequent smokers and predominantly women when compared with the non-mutated NLP group. Data from both groups are shown in table 1.

One patient died in the post-operative setting. During the 18 months follow-up, two patients died within the non-mutated NLP group, while none died in the other group. No differences were found in terms of progression (locally or at distance), progression-free

survival or overall survival between both groups during the follow-up. See Table 3 for details.

DISCUSSION

This study confirms the presence of driver mutations in the normal lung parenchyma cells in the absence of mutations in the primary tumor. This detection of cancer-related mutations in the *EGFR* and *KRAS* oncogenes is consistent with the hypothesis that during the carcinogenesis process multiple cells can gain somatic mutations without necessarily producing a clonal expansion. In our previous study, we confirmed for the first time that the same driver mutation detected in the lung adenocarcinoma was also present in non-tumoral samples but with the limitation that this detection could be secondary to contamination by tumor DNA from blood or tumor cells not detected by the usual histopathological methods. While this limitation was unlikely, it could not be one hundred percent ruled out. After this study, having excluded the presence of mutations in *EGFR* or *KRAS* by highly specific and sensitive techniques in the primary tumor, we are able to confirm that the mutations detected came from the DNA of non-tumoral cells. These findings make us change the way we understand and define a driver-mutation.

The prevalence of *EGFR* and *KRAS* mutations detected in the present study is 9.7%. This prevalence is lower than the one detected in our previous study (21.3%) and in studies of endometriosis (26%) and arteriovenous malformations of the brain (48%). The prevalence is likely to be higher if more extensive molecular studies that included other molecular alterations were carried out. Additionally to having an ambitious hypothesis, we decided to increase the specificity and not include the mutations occurred in later cycles as well as the substitution of L790M in exon 20 that are usually considered as secondary mutations in the final analysis.

In this study we could not find differences in clinical outcomes such as recurrence or disease-free survival, mainly because of the sample size. One patient (33.3%) in the group of the mutated NLP presented progression at 18 months in the form of hepatic metastases; while in the non-mutated NLP group 8 (28.6%) did with a p-value of 0.86.

Unexpectedly, one patient presented three different mutations in the NLP sample that included one in the *EGFR* and two in the *KRAS*. Normally the mutations in *EGFR* and *KRAS* are considered as mutually exclusive mutations in lung cancer. Although this finding is surprising, it does not seem improbable either, since, unlike a tumor, where all the cells come from the clonal expansion of a single malignant cell, the normal lung parenchyma samples contain multiple different cells. Moreover, we believe that this finding further reinforces our hypothesis that molecular changes can occur in multiple cells even without malignancy changes.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. CA Cancer J Clin. 2016;66:7–30.

2. American Cancer Society. Cancer Facts & Figures 2016. Cancer Facts Fig 2016. 2016;1–9.

3. Goldstraw P, Chansky K, Crowley J, Rami-Porta R, Asamura H, Eberhardt WEE, et al. The IASLC lung cancer staging project: Proposals for revision of the TNM stage groupings in the forthcoming (eighth) edition of the TNM Classification for lung cancer. J Thorac Oncol. 2016;11:39–51.

4. Nakagawa T, Okumura N, Ohata K, Igai H, Matsuoka T, Kameyama K. Postrecurrence survival in patients with stage I non-small cell lung cancer. Eur J Cardiothorac Surg. 2008;34:499–504.

5. Martini N, Bains MS, Burt ME, Zakowski MF, McCormack P, Rusch VW, et al. Incidence of local recurrence and second primary tumors in resected stage I lung cancer. J Thorac Cardiovasc Surg. 1995;109:120–9.

6. Chalela R, Curull V, Enríquez C, Pijuan L, Bellosillo B, Gea J. Lung adenocarcinoma: From molecular basis to genome-guided therapy and immunotherapy. Vol. 9, Journal of Thoracic Disease. 2017. p. 2142–58.

7. Stratton MR, Campbell PJ, Futreal PAA. The cancer genome. Nature. 2009;458:719–24.

8. Anglesio MS, Papadopoulos N, Ayhan A, Nazeran TM, Noë M, Horlings HM, et al. Cancer-Associated Mutations in Endometriosis without Cancer. N Engl J Med. 2017;376:1835–48.

9. Nikolaev SI, Vetiska S, Bonilla X, Boudreau E, Jauhiainen S, Rezai Jahromi B, et al. Somatic Activating KRAS Mutations in Arteriovenous Malformations of the Brain. N Engl J Med. 2018;378:250–61.

10. Chalela R, Bellosillo B, Curull V, Longarón R, Pascual-Guardia S, Badenes D, Arriola E, Pijuan L and Gea J. EGFR and KRAS mutations in the non-tumoral lung. Prognosis in patients with adenocarcinoma. Manuscr Submitt Publ.

8.4 ADDITIONAL THESIS MATERIAL-REVIEW

Chalela R, Curull V, Enríquez C, Pijuan L, Bellosillo B, Gea J. Lung adenocarcinoma: from molecular basis to genome-guided therapy and immunotherapy. J Thorac Dis. 2017 Jul;9(7):2142–58. DOI: 10.21037/jtd.2017.06.20

8.5 ADDITIONAL THESIS MATERIAL-EDITORIAL

Chalela R, Gea J, Barreiro E. Immune phenotypes in lung cancer patients with COPD: potential implications for immunotherapy. J Thorac Dis. 2018 Jul;10(Suppl 18):S2186–9. DOI: 10.21037/jtd.2018.06.143

Chapter 9
Discussion

9.1 Main findings

There are several novel findings in the present PhD thesis. As we have mentioned in the first chapters, the field cancerization model includes many events at multiple levels that, if given in a predisposed subject, would generate a clonal expansion. Clearly, since the lung epithelium is broad and have millions of cells directly exposed to risk factors, to think that certain molecular changes could occur in other non-tumoral cells was not a senseless idea, although it was uncertain due to the type of alteration we were looking for. We have detected for the first time the presence of proto-oncogenes mutations in non-cancerous pulmonary cells. In the first paper we identified the same mutation previously detected in resecable lung adenocarcinoma in the histological "healthy" cells. This finding could change the way we define "driver mutation" because with our results it can be assumed that within the process of "cancerization" in normal cells, these somatic mutations can appear in the absence of clonal expansion, at least in the way we define malignancy at present. In all patients included, we defined "normal lung" samples when all cases had tumor-free resection margins and an expert pathologist specialized in lung cancer confirmed the absence of tumor micro-invasion using the traditional methods required for diagnosis of malignancy. Although the presence of some changes in the 'normal cells' is an essential part of our hypothesis, it also implies that their morphological characteristics were still under the limits of normality. Therefore, we believe that an expert pathologist analysis using the standard histopathological methods widely employed in clinical practice, allows us to conclude that all the samples were free of tumor invasion. The presence of circulating-tumor cells from blood vessels as an alternative explanation of our results is certainly possible. However, we think it is unlikely because of the following reasons: the presence of circulating tumoral DNA (ctDNA) and circulating tumoral cells strongly depends on the tumor size, metastasis status and TNM stage (151,152), the specific detection of *EGFR* mutations in the blood of patients with early-stage lung cancer (stages I-II) is relatively low and the detection of *KRAS* by CastPCR in this population is not yet reported although it is assumed to be very low (153). Since our cohort was composed of patients with early-stage lung cancer, with a presumably curative surgery, surgical margins were always negatives, no vessel/lymphatic invasion was detected, and the quantity of blood cells in the sample of non-tumoral lung was probably very low, we can reasonably assume that the mutated DNA we identified did not come from circulating tumoral cells.

The third study was designed to demonstrate that the presence of the driver mutations detected in non-tumoral tissue was definitively not secondary to the presence of circulating tumoral-cells. We identified mutations in *EGFR* or *KRAS* in non-tumoral lung cells of patients without molecular changes in the resected tumor. We use the same approach of the first article to define "normal lung" samples. As mentioned above, although it seemed unlikely, one of the strongest limitations of the first study was that we

could not absolutely rule out that the mutations detected in non-tumoral tissue were due to blood tumoral DNA or circulating malignant cells. With this results, having previously ruled out molecular alterations in the tumor, we can definitively demonstrate that in the process of cancerization, cancer-associated mutations can appear in normal lung cells without clonal expansion. This results are in the line of two recently published articles that evidence for the first time the presence of cancer-related mutations in different nonmalignant disease such as endometriosis and arteriovenous malformations (154,155).

A complete molecular profiling of patients with lung adenocarcinoma is absolutely necessary because of its prognosis/treatment implications and in order to make a more precise diagnosis. Our endoscopy unit is considered of reference in the use of radial-probe ecobronchoscopy guided by fluoroscopy for diagnosis of peripheral lung nodules. We have demonstrated in the second manuscript that brushing specimens obtained by this non-invasive technique and collected in RPMI medium (Roswell Park Memorial Institute medium), a formulation employing a bicarbonate buffering system and alterations in the amounts of amino acids and vitamins used for the culture of human normal and neoplastic cells), provide a useful material for performing molecular testing in patients with peripheral lung adenocarcinoma. Brushing specimens are usually considered to contain limited tumor cellularity and molecular testing may be challenging as an adequate amount of tumoral genomic DNA is not always available (156). However, the high rate (100%) of successful mutational analysis in our study suggests that even though a limited amount of DNA is obtained from brushing samples, it is sufficient to perform PCR-based methods. This results are especially relevant in patients with late-stage lung adenocarcinomas in which we should offer, as a first option, non-invasive tests to avoid excessive discomfort in a population who already have enough problems.

9.2 Prognosis impact

Although the main objective of this thesis was to demonstrate the presence of mutations in healthy tissue, one of the most relevant results we have found are the great impact that the presence of these mutations have on the prognosis of our patients. The results of the first cohort are conclusive with a worse prognosis at one year of follow-up in the patients with the same mutation in the tumor and the non-neoplastic lung parenchyma, specifically in terms of more precocious recurrences and less Disease-Free Survival (DFS). An earlier presence of distant metastases in this group could be understood either as the arrival of already tumoral cells from the primary tumor or the nesting of non-neoplastic cells coming from other parts of the lung, that carrying a driver-mutation, may progress in their new location. The latter possibility is fairly speculative, but could have a huge impact in the way we know or “believe” the recurrences, whether local or at distance, initiate.

Unexpectedly, local recurrence happened only in one patient, meanwhile distant metastases occurred in 60% of the patients with the presence of the same mutation in non-tumoral lung cells. Even more surprising was that the vast majority of these distant metastases occurred in the central nervous system (83% of the affected patients). These results are quite unusual since previous studies report that after curative surgery, more than 50% of the recurrences occurred in pleural space or contralateral lungs, while less than 20% do so initially to the central nervous system (157,158). The interpretation of these results is a challenge. When we compared this recurrence pattern with previous studies that also included early-stage *EGFR* or *KRAS* mutated adenocarcinomas, reinforce our theory that there is a group of patients where histologically healthy cells with the presence of a driver-mutations could migrate through the blood and settle in remote sites and subsequently give way to a new tumor. Probably the passage across the blood-brain barrier and the implantation on the neurological tissue is different in tumor cells and cells without signs of malignancy. While these results are exciting and tempt us to draw risky conclusions, we believe that wider and multicenter studies should be conducted in order to confirm this results and allow us to propose ontological mechanisms in this regard.

9.3 Diagnosis impact.

Considering the high mutational burden that exists in pulmonary adenocarcinoma, until today is considered mandatory to obtain histological samples by biopsy or EBUS (cell blocks) to perform a comprehensive molecular study. However, with the most recent evidence and the results of this doctoral thesis, we can conclude that cytological samples can be valid for the detection of mutations in lung cancer oncogenes. In an additional analysis to that published in our study (manuscript II), we decided to carry out highly sensitive and specific PCR in the non-concordant cytological samples and we were able to detect the somatic mutations previously detected in the histological samples in all the cases. To preserve the bronchial brushing samples in RPMI medium plus the refining of the molecular techniques allow us to make an initial approach with less invasive techniques in the diagnosis of lung adenocarcinoma.

9.4 Molecular techniques used in the study.

The molecular biology is a complex science that is constantly changing and renewing. Several studies are published constantly evaluating the diverse techniques in different scenarios and more sensitive and specific tests that allow us to perform more accurate diagnoses are available. In our studies we used different techniques, mainly real-time PCR using the TheraScreen based on Scorpions® technology plus Sanger sequencing to analyze the *EGFR* and *KRAS* mutational status in the tumor and cytological specimens obtained by brushing. This is the current approach used in our center for detection of *EGFR* and/or *KRAS* in solid malignancies. On the other hand, based in the total absence of previous studies aimed at the detection of driver-mutations in non-tumoral lung tissue, we were expecting a low mutational burden in our samples. Additionally, a high

proportion of our patients also presented histological signs of pulmonary emphysema; thus, we were expecting a low quantity of cell DNA. Consequently, we decided to use the most sensitive and specific test (i.e. TaqMan) to confirm the mutation in the non-tumoral tissue.

TaqMan Mutation Detection Assays is a high sensitivity test for detection of low mutated copies of DNA. It can detect fewer than 10 copies of mutant DNA and have a PCR efficiency of 100% (±10%). Although this technique theoretically allows not only the detection but also the quantification of copies of mutated genes, it is not the best method for the quantification of mutations copies. This is why after the detection of mutations in the non-tumoral tissue using TaqMan, we decided to confirm its presence and quantify the number of mutated copies performing an additional and different PCR technique for this purpose: the Digital PCR, that is a technique where the sample is partitioned to the level of single molecules and then PCR amplification is performed (159). In all cases we confirmed the mutations and in the cases where *EGFR* was detected, a mean of 0.20% of mutated copies with respect to total copies of the gene were identified. While for *KRAS*, a mean of 0.08% of mutated copies were identified. This quantification allowed us to confirm the excellent performance of these two techniques (TaqMan and Digital PCR) in the detection of mutations in samples with very low load of mutated DNA since we were able to detect a minimum of copies of up to 0.02%.

9.5 Limitations of the present PhD Thesis.

In the present doctoral thesis there are several limitations that depend especially on the methodology used and ethical aspects. Despite making a rigorous design, with quite clear inclusion criteria and using the furthermost non-tumoral lung tissue from the primary tumor, most of the samples analyzed came from the same or adjacent pulmonary lobe. Although it would be ideal to be able to demonstrate that the driver mutations can occur in contralateral lung cells, ethically it did not seem right to consider invasive techniques in patients with a disease that intrinsically has a great emotional impact and requires multiple invasive examinations for its diagnosis and treatment. Taking these aspects into account, we decided to use the samples as far as possible from the primary tumor with very strict criteria to verify the absence of tumor cells and minimize these limitation.

On the other hand, the results that we are presenting are very interesting and probably would impact patients care, but in part they are in the process of being published. At present, patients who undergo curative surgery for adenocarcinoma are the minority, so the recruitment of patients undergoing surgical resection and with mutations in the tumor is labored. We also followed-up for at least 12 month all the patients included in our cohorts, so this contributed with more time in the development of our studies. The time factor is an intrinsic factor in all doctoral theses but above all in clinical and translational projects.

Chapter 10
Conclusions And Future Perspectives

10.1 Conclusions

1. Cancer-related mutations also known as driver mutations, specifically *EGFR* or *KRAS,* can appear in normal lung cells during the cancerization process of the pulmonary epithelium. These molecular alterations can be present in lungs of patients with previously resected adenocarcinoma regardless of the mutational status in the tumor.

2. The presence of the same driver mutations in non-tumoral lung cells worsen the prognosis of patients with early-stage lung adenocarcinoma in terms of distant recurrences and disease-free survival. In this patients, metastasis to the central nervous system occur, unexpectedly, in 83% during the first year.

3. TaqMan Mutation Detection Assays can identify mutations in samples with low load of mutated DNA and is able to detect a minimum of copies of up to 0.02% % of mutated copies with respect to total copies of the gene.

4. Brushing specimens conserved in RPMI medium and obtained by R-EBUS plus fluoroscopy-guided bronchoscopy are useful for detecting *EGFR* and *KRAS* mutational status in patients with peripheral lung adenocarcinoma using DNA-based RT-PCR and Sanger sequencing.

10.2 Future Perspectives

This project contribute with several novel conclusions to both the knowledge of molecular mechanisms occurring during the process of cancerization, and the diagnosis of lung malignancy. The first and most important step following all this findings is to design a multicenter validation cohort, including a wider molecular profile (*EGFR, KRAS* and *BRAF* mutations, *EML4-ALK* rearrangement and *ROS1* fusions) in order to make deeper and more precise conclusions, as well as to design a similar cohort including patients without cancer but with risk factors (e.g smokers and/or emphysema) to demonstrate molecular alterations in the absence of malignancy. Simultaneously we are in developing an ambitious project to perform multi-level molecular analysis that include tumor, lung tissue, blood, urine and nasal epithelium in patients with and without lung adenocarcinoma.

The future of molecular analysis goes beyond cancer, since we also hypothesized that certain mutations can occur even before the neoplasm appears. An exciting scenario would be the early detection of genomic somatic changes in high-risk patients.

References

1. Chalela R, Curull V, Enríquez C, Pijuan L, Bellosillo B, Gea J. Lung adenocarcinoma: From molecular basis to genome-guided therapy and immunotherapy. J Thorac Dis. 2017;99:2142-2158
2. Siegel RL, Miller KD, Jemal A. Cancer statistics. CA Cancer J Clin. 2016;66:7–30.
3. Galceran J, Ameijide A, Carulla M, Mateos A, Quirós JR, Rojas D, et al. Cancer incidence in Spain, 2015. Clin Transl Oncol. 2017;19:799–825.
4. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol. 2011;6:244–85.
5. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
6. Gibelin C, Couraud S. Somatic alterations in lung cancer: Do environmental factors matter? Lung Cancer. 2016;100:45–52.
7. Ruano-Ravina A, Fernández-Villar A, Barros-Dios JM. Radón residencial y riesgo de cáncer de pulmón en nunca fumadores. Arch Bronconeumol. 2017; 53:475-476
8. Travis WD, Brambilla E, Müller-Hermelink HK, Harris C. World Health Organization classification of tumours; tumours of lung, pleura, thymus and heart. World Heal Organ Classif tumours. 2004;9–122.
9. Sanchez-Salcedo P, Berto J, De-Torres JP, Campo A, Alcaide AB, Bastarrika G, et al. Lung cancer screening: fourteen year experience of the Pamplona Early Detection Program (P-IELCAP). Arch Bronconeumol. 2014;51:169–76.
10. American Cancer Society. Cancer Facts & Figures 2016. Cancer Facts Fig 2016. 2016;1–9.
11. Goldstraw P, Chansky K, Crowley J, Rami-Porta R, Asamura H, Eberhardt WEE, et al. The IASLC lung cancer staging project: Proposals for revision of the TNM stage groupings in the forthcoming (eighth) edition of the TNM Classification for lung cancer. J Thorac Oncol. 2016;11:39–51.
12. Cancer T, Atlas G, Collisson E a., Campbell JD, Brooks AN, Berger AH, et al. Comprehensive molecular profiling of lung adenocarcinoma. Nature. 2014;511:543–50.
13. Kim HS, Mitsudomi T, Soo RA, Cho BC. Personalized therapy on the horizon for squamous cell carcinoma of the lung. Lung Cancer. 2013;80:249–55.
14. Hecht SS. Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst. 1999;91:1194–210.
15. International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Tob Smoke Involuntary Smok. 2004;83:35–102
16. Doll R, Hill AB. The mortality of doctors in relation to their smoking habits; a preliminary report. Br Med J. 1954;1:1451–5.
17. Doll R, Peto R. Mortality in relation to smoking: 20 years' observations on male British doctors. Br Med J. 1976;2:1525–36.

18. Doll R, Peto R, Wheatley K, Gray R, Sutherland I. Mortality in relation to smoking: 40 years' observations on male British doctors. BMJ. 1994;309:901–11.
19. Doll R, Peto R, Boreham J, Sutherland I. Mortality in relation to smoking: 50 years' observations on male British doctors. BMJ. 2004;328:1519.
20. US Surgeon General's Advisory Committee on Smoking and Health. Smoking and health; report of the advisory committee to the Surgeon General of the Public Health Service. Washington, DC: US Depart- ment of Health, Education, and Welfare; 1964, pp. 23-40.
21. McLaughlin JK, Hrubec Z, Blot WJ, Fraumeni JF. Smoking and cancer mortality among U.S. veterans: a 26-year follow-up. Int J cancer. 1995;60:190–3.
22. Rivera GA, Wakelee H. Lung Cancer in Never Smokers. Adv Exp Med Biol. 2016;893:43–57.
23. Wu X, Amos CI, Zhu Y, Zhao H, Grossman BH, Shay JW, et al. Telomere dysfunction: a potential cancer predisposition factor. J Natl Cancer Inst. 2003;95:1211–8.
24. Wenzlaff AS, Cote ML, Bock CH, Land SJ, Santer SK, Schwartz DR, et al. CYP1A1 and CYP1B1 polymorphisms and risk of lung cancer among never smokers: a population-based study. Carcinogenesis. 2005;26:2207–12.
25. Park JY, Park JM, Jang JS, Choi JE, Kim KM, Cha SI, et al. Caspase 9 promoter polymorphisms and risk of primary lung cancer. Hum Mol Genet. 2006;15:1963–71.
26. Kleihues P, Schäuble B, zur Hausen A, Estève J, Ohgaki H. Tumors associated with p53 germline mutations: a synopsis of 91 families. Am J Pathol. 1997;150:1–13.
27. Okazaki I, Ishikawa S, Sohara Y. Genes associated with succeptibility to lung adenocarcinoma among never smokers suggest the mechanism of disease. Anticancer Res. 2014;34:5229–40.
28. Amos CI, Wu X, Broderick P, Gorlov IP, Gu J, Eisen T, et al. Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. Nat Genet. 2008;40:616–22.
29. Wang Y, Broderick P, Webb E, Wu X, Vijayakrishnan J, Matakidou A, et al. Common 5p15.33 and 6p21.33 variants influence lung cancer risk. Nat Genet. 2008;40:1407–9.
30. Yang IA, Holloway JW, Fong KM. Genetic susceptibility to lung cancer and co-morbidities. J Thorac Dis. 2013;5:S454-62
31. Wang H-M, Zhang X-Y, Jin B. TERT genetic polymorphism rs2736100 was associated with lung cancer: a meta-analysis based on 14,492 subjects. Genet Test Mol Biomarkers. 2013;17:937–41.
32. Li T, Xian Y, Tian T, Zhuang X, Chu M. New evidence of TERT rs2736098 polymorphism and cancer risk: an updated meta-analysis. J BUON. 2016;21:491–7.
33. Campa D, Rizzato C, Stolzenberg-Solomon R, Pacetti P, Vodicka P, Cleary SP, et al. TERT gene harbors multiple variants associated with pancreatic cancer susceptibility. Int J cancer. 2015;137:2175–83.
34. Shete S, Hosking FJ, Robertson LB, Dobbins SE, Sanson M, Malmer B, et al. Genome-wide association study identifies five susceptibility loci for glioma. Nat Genet. 2009;41:899–904.
35. Zienolddiny S, Skaug V, Landvik NE, Ryberg D, Phillips DH, Houlston R,

et al. The TERT-CLPTM1L lung cancer susceptibility variant associates with higher DNA adduct formation in the lung. Carcinogenesis. 2009;30:1368–71.
36. Lochhead P, Chan AT, Nishihara R, Fuchs CS, Beck AH, Giovannucci E, et al. Etiologic field effect: reappraisal of the field effect concept in cancer predisposition and progression. Mod Pathol. 2015;28:14–29.
37. Lee JJ, Liu D, Lee JS, Kurie JM, Khuri FR, Ibarguen H, et al. Long-term impact of smoking on lung epithelial proliferation in current and former smokers. J Natl Cancer Inst. 2001;93:1081–8.
38. Holliday R. The inheritance of epigenetic defects. Science. 1987;238:163–70.
39. Barreiro E, Gea J. Epigenetics and muscle dysfunction in chronic obstructive pulmonary disease. Transl Res. 2015;165:61–73.
40. Daugaard I, Dominguez D, Kjeldsen TE, Kristensen LS, Hager H, Wojdacz TK, et al. Identification and validation of candidate epigenetic biomarkers in lung adenocarcinoma. Sci Rep. 2016;6:35807.
41. Ansari J, Shackelford RE, El-Osta H. Epigenetics in non-small cell lung cancer: from basics to therapeutics. Transl Lung Cancer Res. 2016;5:155–71.
42. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57–70.
43. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol. 2007;35:495–516.
44. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007;26:1324–37.
45. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metastasis Rev. 2010;29:309–16.
46. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell. 2010;140:883–99.
47. Qian B-Z, Pollard JW. Macrophage Diversity Enhances Tumor Progression and Metastasis. Cell. 2010;141:39–51.
48. Karnoub AE, Weinberg RA. Chemokine networks and breast cancer metastasis. Breast Dis. 2006-2007;26:75-85
49. Berdasco M, Esteller M. Aberrant Epigenetic Landscape in Cancer: How Cellular Identity Goes Awry. Dev Cell. 2010;19:698–711.
50. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;128:683–92.
51. Stratton MR, Campbell PJ, Futreal PAA. The cancer genome. Nature 2009;458:719–24.
52. Gaughran SJ, Pless E, Stearns SC. How elephants beat cancer. Elife. 2016;5:e21864
53. Tamborero D, Gonzalez-Perez A, Perez-Llamas C, Deu-Pons J, Kandoth C, Reimand J, et al. Comprehensive identification of mutational cancer driver genes across 12 tumor types. Sci Rep. 2013;3:2650.
54. Chalela R, Bellosillo B, Curull V, Pijuan L GJ. Prevalence of molecular changes in resected pulmonary adenocarcinomas. Eur Respir J. 2016;48:PA2853.
55. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. Cancer Discov. 2012;2:401-4
56. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al.

Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. Sci Signal. 2013;6:pl1-pl1. Available from: http://stke.sciencemag.org/cgi/doi/10.1126/scisignal.2004088
57. Tsiambas E, Lefas AY, Georgiannos SN, Ragos V, Fotiades PP, Grapsa D, et al. EGFR gene deregulation mechanisms in lung adenocarcinoma: A molecular review. Pathol - Res Pract. 2016;212:672–7.
58. Antonicelli A, Cafarotti S, Indini A, Galli A, Russo A, Cesario A, et al. Egfr-targeted therapy for non-small cell lun cancer: Focus on EGFR oncogenic mutation. Int J Med Sci. 2013;10:320–30.
59. Devarakonda S, Morgensztern D, Govindan R. Genomic alterations in lung adenocarcinoma. Lancet Oncol. 2015;16:e342–51.
60. Sharma S V., Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer. 2007;7:169–81.
61. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene . 2006;366:2–16.
62. Cheng L, Zhang S, Alexander R, Yao Y, MacLennan GT, Pan C, et al. The landscape of EGFR pathways and personalized management of non-small-cell lung cancer. Future Oncol. 2011;7:519–41.
63. Ha SY, Choi S-J, Cho JH, Choi HJ, Lee J, Jung K, et al. Lung cancer in never-smoker Asian females is driven by oncogenic mutations, most often involving EGFR. Oncotarget. 2015;6:5465–74.
64. Tomita M, Ayabe T, Chosa E, Kawagoe K, Nakamura K. Epidermal growth factor receptor mutations in Japanese men with lung adenocarcinomas. Asian Pac J Cancer Prev. 2014;15:10627–30.
65. Nishii T, Yokose T, Miyagi Y, Daigo Y, Isaka T, Furumoto H, et al. Prognostic value of EGFR mutations in surgically resected pathological stage I lung adenocarcinoma. Asia Pac J Clin Oncol. 2017;13:e204-e211
66. Soria J-C, Mok TS, Cappuzzo F, Jänne PA. EGFR-mutated oncogene-addicted non-small cell lung cancer: Current trends and future prospects. Cancer Treat Rev. 2012;38:416–30.
67. Zhu J, Zhong W, Zhang G, Li R, Zhang X, Guo A, et al. Better survival with EGFR exon 19 than exon 21 mutations in gefitinib-treated non-small cell lung cancer patients is due to differential inhibition of downstream signals. Cancer Lett. 2008;265:307–17.
68. Hirsch FR, Suda K, Wiens J, Bunn PA. New and emerging targeted treatments in advanced non-small-cell lung cancer. Lancet (London, England). 2016;388:1012–24.
69. Hirsch FR, Scagliotti G V, Mulshine JL, Kwon R, Curran WJ, Wu Y-L, et al. Lung cancer: current therapies and new targeted treatments. Lancet (London, England). 2016;6736:1–13.
70. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer. 2003;3:11–22.
71. Santos E, Martin-Zanca D, Reddy EP, Pierotti MA, Della Porta G, Barbacid M. Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue of the same patient. Science. 1984;223:661–4.
72. Lee B, Lee T, Lee S, Choi Y, Han J. Clinicopathologic characteristics of EGFR, KRAS, and ALK alterations in 6,595 lung cancers. Oncotarget. 2016; 7:23874-84
73. Dacic S, Shuai Y, Yousem S, Ohori P, Nikiforova M. Clinicopathological

predictors of EGFR/KRAS mutational status in primary lung adenocarcinomas. Mod Pathol. 2010;23:159–68.
74. Rekhtman N, Ang DC, Riely GJ, Ladanyi M, Moreira AL. KRAS mutations are associated with solid growth pattern and tumor-infiltrating leukocytes in lung adenocarcinoma. Mod Pathol. 2013;26:1307–19.
75. Kadota K, Sima CS, Arcila ME, Hedvat C, Kris MG, Jones DR, et al. KRAS Mutation Is a Significant Prognostic Factor in Early-stage Lung Adenocarcinoma. Am J Surg Pathol. 2016;40:1579–90.
76. Califano R, Landi L, Cappuzzo F. Prognostic and Predictive Value of K-RAS Mutations in Non-Small Cell Lung Cancer. Drugs. 2012;72:28–36.
77. Meng D, Yuan M, Li X, Chen L, Yang J, Zhao X, et al. Prognostic value of K-RAS mutations in patients with non-small cell lung cancer: A systematic review with meta-analysis. Lung Cancer. 2013;81:1–10.
78. Nadal E, Chen G, Prensner JR, Shiratsuchi H, Sam C, Zhao L, et al. KRAS-G12C mutation is associated with poor outcome in surgically resected lung adenocarcinoma. J Thorac Oncol. 2014;9:1513–22.
79. Gautschi O, Milia J, Cabarrou B, Bluthgen M-V, Besse B, Smit EF, et al. Targeted Therapy for Patients with BRAF-Mutant Lung Cancer Results from the European EURAF Cohort. J Thorac Oncol. 2009;10:1451–7.
80. Li Z, Jiang L, Bai H, Wang Z, Zhao J, Duan J, et al. Prevalence and clinical significance of BRAF V600E in Chinese patients with lung adenocarcinoma. Thorac Cancer. 2015;6:269–74.
81. Tissot C, Couraud S, Tanguy R, Bringuier P-P, Girard N, Souquet P-J. Clinical characteristics and outcome of patients with lung cancer harboring BRAF mutations. Lung Cancer. 2016;91:23–8.
82. Marchetti A, Felicioni L, Malatesta S, Grazia Sciarrotta M, Guetti L, Chella A, et al. Clinical Features and Outcome of Patients With Non-Small-Cell Lung Cancer Harboring BRAF Mutations. J Clin Oncol. 2011;29:3574–9.
83. Rasmussen S a, Friedman JM. NF1 gene and neurofibromatosis 1. Am J Epidemiol. 2000;151:33–40.
84. Cichowski K, Shih TS, Schmitt E, Santiago S, Reilly K, McLaughlin ME, et al. Mouse models of tumor development in neurofibromatosis type 1. Science. 1999;286:2172–6.
85. Redig AJ, Capelletti M, Dahlberg SE, Sholl LM, Mach SL, Fontes C, et al. Clinical and molecular characteristics of NF1 mutant lung cancer. Clin Cancer Res. 2016;22:1–3.
86. Cipriani NA, Abidoye OO, Vokes E, Salgia R. MET as a target for treatment of chest tumors. Lung Cancer. 2009;63:169–79.
87. Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev. 2003;22:309–25.
88. Landi L, Minuti G, D'Incecco A, Cappuzzo F. Targeting c-MET in the battle against advanced nonsmall-cell lung cancer. Curr Opin Oncol. 2013;25:130–6.
89. Robinson KW, Sandler AB. The Role of MET Receptor Tyrosine Kinase in Non-Small Cell Lung Cancer and Clinical Development of Targeted Anti-MET Agents. Oncologist. 2013;18:115–22.
90. Álvarez FV, Trueba IM, Sanchis JB, López-Rodó LM, Rodríguez Suárez PM, de Cos Escuín JS, et al. Recommendations of the Spanish Society of Pneumology and Thoracic Surgery on the diagnosis and treatment of non-

small-cell lung cancer. Arch Bronconeumol. 2016;52:2–62.
91. Korpanty GJ, Graham DM, Vincent MD, Leighl NB. Biomarkers that currently effect clinical practice in lung cancer: EGFR, ALK, MET, ROS-1 and KRAS. Front Oncol. 2014;4:1–26.
92. Kerr KM. Precision medicine in NSCLC and pathology : how does ALK fi t in the pathway ?. Ann Oncol. 2016;27:16-24
93. Le T, Gerber DE. ALK alterations and inhibition in lung cancer. Semin Cancer Biol. 2017;42:81-88
94. Tao H, Cai Y, Shi L, Tang J, Liu Z, Wang Z, et al. Analysis of clinical characteristics and prognosis of patients with anaplastic lymphoma kinase-positive and surgically resected lung adenocarcinoma. Thorac cancer. 2017;8:8-15
95. Incharoen P, Reungwetwattana T, Saowapa S, Kamprerasart K, Pangpunyakulchai D, Arsa L, et al. ALK-rearranged pulmonary adenocarcinoma in Thai Patients: From diagnosis to treatment efficacy. World J Surg Oncol. 2016;14:139.
96. Camidge DR, Kono SA, Flacco A, Tan A-C, Doebele RC, Zhou Q, et al. Optimizing the Detection of Lung Cancer Patients Harboring Anaplastic Lymphoma Kinase (ALK) Gene Rearrangements Potentially Suitable for ALK Inhibitor Treatment. Clin Cancer Res. 2010;16:5581–90.
97. Kohno T, Nakaoku T, Tsuta K, Tsuchihara K, Matsumoto S, Yoh K, et al. Beyond ALK-RET, ROS1 and other oncogene fusions in lung cancer. Transl Lung Cancer Res. 2015;4:156–64.
98. Shaw AT, Ou S-HI, Bang Y-J, Camidge DR, Solomon BJ, Salgia R, et al. Crizotinib in *ROS1* -Rearranged Non–Small-Cell Lung Cancer. N Engl J Med. 2014;371:1963–71.
99. Davies KD, Doebele RC. Molecular Pathways: ROS1 Fusion Proteins in Cancer. Clin Cancer Res. 2013;19:4040–5.
100. Villar Álvarez F, Muguruza Trueba I, Belda Sanchis J, Molins López-Rodó L, Rodríguez Suárez PM, Sánchez de Cos Escuín J, et al. Executive summary of the SEPAR recommendations for the diagnosis and treatment of non-small cell lung cancer. Arch Bronconeumol. 2016;52:378–88.
101. Lazarus DR, Ost DE. How and when to use genetic markers for nonsmall cell lung cancer. Curr Opin Pulm Med. 2013;19:331–9.
102. Planchard D, Besse B, Groen HJM, Souquet P-J, Quoix E, Baik CS, et al. Dabrafenib plus trametinib in patients with previously treated BRAFV600E-mutant metastatic non-small cell lung cancer: an open-label, multicentre phase 2 trial. Lancet Oncol. 2016;17:984–93.
103. Monsó E, Montuenga LM, Sánchez de Cos, Villena C. Biological Marker Analysis as Part of The CIBERES-RTIC Cancer-SEPAR Strategic Project on Lung Cancer. Arch Bronconeumol. 2014;51:462–7.
104. Fernandez-Bussy S, Labarca G, Pires Y, Caviedes I, Burotto M. Análisis moleculares de EGFR, mutación de resistencia al EGFR, ALK y ROS1 en muestras obtenidas mediante PATB-USEB en Chile. Arch Bronconeumol. 2017;53:172-174
105. Jeyabalan A, Bhatt N, Plummeridge M, Medford A. Adequacy of endobronchial ultrasound‑guided transbronchial needle aspiration samples processed as histopathological samples for genetic mutation analysis in lung adenocarcinoma. Mol Clin Oncol. 2015;4:119–25.

106. Trisolini R, Cancellieri A, Tinelli C, de Biase D, Valentini I, Casadei G, et al. Randomized Trial of Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration With and Without Rapid On-site Evaluation for Lung Cancer Genotyping. Chest. 2015;148:1430–7.
107. Bravaccini S, Tumedei MM, Ulivi P, Zoli W, Calistri D, Candoli P, et al. *ALK* translocation detection in non-small cell lung cancer cytological samples obtained by TBNA or EBUS-TBNA. Cytopathology. 2016;27:103–7.
108. Chalela R, Sánchez-Font A, Domínguez-Álvarez M, Badenes-Bonet D, Pijuan L, Curull V. Role of endobronchial ultrasound-guided transbronchial needle aspiration in the diagnosis of mediastinal tuberculosis. Med Clínica (English Ed). 2016;146:532–5.
109. Hopkins E, Moffat D, Parkinson I, Robinson P, Jersmann H, Dougherty B, et al. Cell block samples from endobronchial ultrasound transbronchial needle aspiration provide sufficient material for ancillary testing in lung cancer—a quaternary referral centre experience. J Thorac Dis. 2016;8:2544–50.
110. Reynolds JP, Tubbs RR, Minca EC, MacNamara S, Almeida FA, Ma PC, et al. EGFR mutational genotyping of liquid based cytology samples obtained via fine needle aspiration (FNA) at endobronchial ultrasound of non-small cell lung cancer (NSCLC). Lung Cancer. 2014;86:158–63.
111. Travis, W.D., Brambilla, E., Burke, A.P., Marx, A., Nicholson AG. *WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart*. 4th edition. Geneve: 2015.
112. Travis WD. Classification of lung cancer. Semin Roentgenol. 2011;46:178–86.
113. Travis WD. The 2015 WHO classification of lung tumors. Pathologe. 2014;35:188–188.
114. Travis WD, Rekhtman N. Pathological diagnosis and classification of lung cancer in small biopsies and cytology: strategic management of tissue for molecular testing. Semin Respir Crit Care Med. 2011;32:22–31.
115. Cagle PT, Allen TC, Bernicker EH, Ge Y, Haque A, Barrios R. Impact of recent developments in lung cancer on the practice of pathology. Arch Pathol Lab Med. 2016;140(4):322–5.
116. Rami-Porta R, Bolejack V, Giroux DJ, Chansky K, Crowley J, Asamura H, et al. The IASLC Lung Cancer Staging Project: The New Database to Inform the Eighth Edition of the TNM Classification of Lung Cancer. J Thorac Oncol. 2014;9:1618–24.
117. Pater JL, Loeb M. Nonanatomic prognostic factors in carcinoma of the lung. A multivariate analysis. Cancer. 1982;50:326–31.
118. Howington JA, Blum MG, Chang AC, Balekian AA, Murthy SC. Treatment of Stage I and II Non-small Cell Lung Cancer. Chest. 2013;143:e278S–e313S.
119. Ramnath N, Dilling TJ, Harris LJ, Kim AW, Michaud GC, Balekian AA, et al. Treatment of Stage III Non-small Cell Lung Cancer. Chest. 2013;143:e314S–e340S.
120. Socinski MA, Evans T, Gettinger S, Hensing TA, VanDam Sequist L, Ireland B, et al. Treatment of Stage IV Non-small Cell Lung Cancer. Chest. 2013;143:e341S–e368S.
121. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, 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;13:239–46.
122. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol. 2010;11:121–8.
123. Sequist L V., Yang JC-H, Yamamoto N, O'Byrne K, Hirsh V, Mok T, et al. Phase III Study of Afatinib or Cisplatin Plus Pemetrexed in Patients With Metastatic Lung Adenocarcinoma With *EGFR* Mutations. J Clin Oncol. 2013;31:3327–34.
124. Wu Y-L, Zhou C, Liam C-K, Wu G, Liu X, Zhong Z, et al. First-line erlotinib versus gemcitabine/cisplatin in patients with advanced *EGFR* mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study. Ann Oncol. 2015;26:1883–9.
125. Yang JC-H, Sequist L V, Geater SL, Tsai C-M, Mok TSK, Schuler M, et al. Clinical activity of afatinib in patients with advanced non-small-cell lung cancer harbouring uncommon EGFR mutations: a combined post-hoc analysis of LUX-Lung 2, LUX-Lung 3, and LUX-Lung 6. Lancet Oncol. 2015;16:830–8.
126. Mayor S. Osimertinib effective in EGFR T790M-positive lung cancer. Lancet Oncol. 2017;18:e9.
127. Liao B-C, Lin C-C, Lee J-H, Yang JC-H. Update on recent preclinical and clinical studies of T790M mutant-specific irreversible epidermal growth factor receptor tyrosine kinase inhibitors. J Biomed Sci. 2016;23:86.
128. Shaw AT, Kim D-W, Nakagawa K, Seto T, Crinó L, Ahn M-J, et al. Crizotinib versus Chemotherapy in Advanced *ALK* -Positive Lung Cancer. N Engl J Med. 2013;368:2385–94.
129. Chuang JC, Neal JW. Crizotinib as first line therapy for advanced ALK-positive non-small cell lung cancers. Transl lung cancer Res. 2015;4:639–41.
130. Shaw AT, Engelman JA. Ceritinib in *ALK* -Rearranged Non–Small-Cell Lung Cancer. N Engl J Med. 2014;370:2537–9.
131. Gadgeel SM, Gandhi L, Riely GJ, Chiappori AA, West HL, Azada MC, 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-002JG): results from the dose-finding portion of a phase 1/2 study. Lancet Oncol. 2014;15:1119–28.
132. Shaw AT, Gandhi L, Gadgeel S, Riely GJ, Cetnar J, West H, et al. Alectinib in ALK-positive, crizotinib-resistant, non-small-cell lung cancer: a single-group, multicentre, phase 2 trial. Lancet Oncol. 2016;17:234–42.
133. Drilon A, Cappuzzo F, Ou S-HI, Camidge DR. Targeting MET in Lung Cancer: Will Expectations Finally Be MET? J Thorac Oncol. 2017;12:15–26.
134. Pardoll D. *Cancer Immunotherapy with Vaccines and Checkpoint Blockade*. In: Mendelsohn J, Howley P, Israel M, Gray J and Thompson C*The Molecular Basis of Cancer*: Fourth Edition. NY: Saunders; 2014.
135. Naylor EC, Desani JK, Chung PK. Targeted Therapy and Immunotherapy for Lung Cancer. Surg Oncol Clin N Am. 2016;25:601–9.

136. Du L, Herbst RS, Morgensztern D. Immunotherapy in Lung Cancer. Hematol Oncol Clin North Am. 2017;31:131–41.
137. Vansteenkiste J, Zielinski M, Linder A, Dahabreh J, Gonzalez EE, Malinowski W, et al. Adjuvant MAGE-A3 immunotherapy in resected non-small-cell lung cancer: phase II randomized study results. J Clin Oncol. 2013;31:2396–403.
138. Butts C, Socinski MA, Mitchell PL, Thatcher N, Havel L, Krzakowski M, et al. Tecemotide (L-BLP25) versus placebo after chemoradiotherapy for stage III non-small-cell lung cancer (START): a randomised, double-blind, phase 3 trial. Lancet Oncol. 2014;15:59–68.
139. Nemunaitis J, Nemunaitis M, Senzer N, Snitz P, Bedell C, Kumar P, et al. Phase II trial of Belagenpumatucel-L, a TGF-beta2 antisense gene modified allogeneic tumor vaccine in advanced non small cell lung cancer (NSCLC) patients. Cancer Gene Ther. 2009;16:620–4.
140. Khanna P, Blais N, Gaudreau P-O, Corrales-Rodriguez L. Immunotherapy Comes of Age in Lung Cancer. Clin Lung Cancer. 2016; 18:13-22
141. P. Zatloukal, D. S. Heo, K. Park, J. Kang, C. Butts, D. Bradford, S. Graziano, B. Huang DH. Randomized phase II clinical trial comparing tremelimumab (CP-675,206) with best supportive care (BSC) following first-line platinum-based therapy in patients (pts) with advanced non-small cell lung cancer (NSCLC). In: 2009 ASCO Annual Meeting. 2009:p.8071.
142. Planchard D, Yokoi T, McCleod MJ, Fischer JR, Kim YC, Ballas M, et al. A Phase III Study of Durvalumab (MEDI4736) with or Without Tremelimumab for Previously Treated Patients with Advanced NSCLC: Rationale and Protocol Design of the ARCTIC Study. Clin Lung Cancer. 2016;17:232–236e1.
143. Scott Joseph Antonia, Sarah B. Goldberg, Ani Sarkis Balmanoukian, Rachel E. Sanborn, Keith Steele, Rajesh Narwal, Paul B. Robbins, Yu Gu, Joyson Joseph Karakunnel, Naiyer A. RizviScott Joseph Antonia, Sarah B. Goldberg, Ani Sarkis Balmanoukian, Rachel E. NAR. Phase Ib study of MEDI4736, a programmed cell death ligand-1 (PD-L1) antibody, in combination with tremelimumab, a cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) antibody, in patients (pts) with advanced NSCLC. J Clin Oncol. 2015;33:A3014).
144. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature. 2011;480:480–9.
145. Gettinger SN, Horn L, Gandhi L, Spigel DR, Antonia SJ, Rizvi NA, et al. Overall Survival and Long-Term Safety of Nivolumab (Anti-Programmed Death 1 Antibody, BMS-936558, ONO-4538) in Patients With Previously Treated Advanced Non-Small-Cell Lung Cancer. J Clin Oncol. 2015;33:2004–12.
146. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non–Small-Cell Lung Cancer. N Engl J Med. 2015;373:1627–39.
147. Herbst RS, Baas P, Kim D-W, Felip E, Pérez-Gracia JL, Han J-Y, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet (London, England). 2016;387:1540–50.
148. Langer CJ, Gadgeel SM, Borghaei H, Papadimitrakopoulou VA, Patnaik A, Powell SF, et al. Carboplatin and pemetrexed with or without

pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. Lancet Oncol. 2016;17:1497–508.
149. Rittmeyer A, Barlesi F, Waterkamp D, Park K, Ciardiello F, von Pawel J, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet. 2017;389:255–65.
150. Mark NM, Kargl J, Busch SE, Yang GHY, Metz HE, Zhang H, et al. Chronic Obstructive Pulmonary Disease Alters Immune Cell Composition and Immune Checkpoint Inhibitor Efficacy in Non-Small Cell Lung Cancer. Am J Respir Crit Care Med. 2018;197:325–36.
151. Bettegowda. Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies. Sci Transl Med. 2014;6:p224.
152. Chen KZ, Lou F, Yang F, Zhang JB, Ye H, Chen W, et al. Circulating Tumor DNA Detection in Early-Stage Non-Small Cell Lung Cancer Patients by Targeted Sequencing. Sci Rep. 2016;6:31985.
153. Pérez-Callejo 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;5:455–65.
154. Nikolaev SI, Vetiska S, Bonilla X, Boudreau E, Jauhiainen S, Rezai Jahromi B, et al. Somatic Activating *KRAS* Mutations in Arteriovenous Malformations of the Brain. N Engl J Med. 2018;378:250–61.
155. Anglesio MS, Papadopoulos N, Ayhan A, Nazeran TM, Noë M, Horlings HM, et al. Cancer-Associated Mutations in Endometriosis without Cancer. N Engl J Med. 2017;376:1835–48.
156. Aisner DL, Marshall CB. Molecular pathology of non-small cell lung cancer: A practical guide. American Journal of Clinical Pathology. 2012;138:332–46.
157. Lou F, Huang J, Sima CS, Dycoco J, Rusch V, Bach PB. Patterns of recurrence and second primary lung cancer in early-stage lung cancer survivors followed with routine computed tomography surveillance. J Thorac Cardiovasc Surg. 2013;145:75-81
158. Mizuno T, Arimura T, Kuroda H, Sakakura N, Yatabe Y, Sakao Y. Current outcomes of postrecurrence survival in patients after resection of non-small cell lung cancer. J Thorac Dis. 2018;10:1788–96.
159. Vogelstein B, Kinzler KW. Digital PCR. Genetics. 1999;96:9236–41.