EPIDEMIOLOGY AND CLINICAL RELEVANCE OF SUBTYPE-SPECIFIC KRAS AND EGFR MUTATIONS IN LUNG ADENOCARCINOMA

PhD Dissertation

Zoltán Lohinai, MD

Semmelweis University
Clinical Medicine PhD School

Supervisors: Dr. Balázs Hegedűs, PhD
Dr. Balázs Döme, MD, PhD

Official reviewers:
Dr. Zsolt István Komlósi MD, PhD
Dr. Nóra Bittner MD, PhD

Head of the Final Examination Committee:
Dr. Gabriella Lengyel, MD, PhD
Members of the Final Examination Committee:
Dr. György Böszörményi Nagy, MD, PhD
Dr. Gabriella Gálffy, MD, PhD

Budapest
2016

TABLE OF CONTENTS

ABBREVIATIONS

A - adenine
AIS - in situ pulmonary adenocarcinoma
AKT - protein kinase B (PKB), also known as AKT
ALK - anaplastic lymphoma kinase
ANOVA - analysis of variance
Arg - Arginine
ARMS - amplification refractory mutation system
A-rule - glycine to alanine transitions
ATCC - American Type Culture Collection
B7 - type of peripheral membrane protein found on activated antigen presenting cells (APC)
BAC - bronchioloalveolar carcinoma
BRAF/B-Raf – oncogen/protein; v-raf (viral rapidly accelerated fibrosarcoma) murine sarcoma viral oncogene homolog B1
BstNI or BglI - restriction enzymes
C - cytosine
CI - confidence intervals
COSMIC - Catalogue of Somatic Mutations in Cancer
CR- complete response
CT - computed tomography
CTLA-4 - cytotoxic T-lymphocyte-associated protein 4
Del - deletion
DMEM – Dulbecco's Modified Eagle's Medium
DNA – deoxyribonucleic acid
dNTP - deoxynucleotide triphosphates
EBUS - endobronchial ultrasound
ECOG PS - Eastern Cooperative Oncology Group performance status
EGF / EGFR – epidermal growth factor / epidermal growth factor receptor
EGFR-TKI - epidermal growth factor receptor tyrosine kinase inhibitor
EMA - European Medicines Agency
EML4-ALK Echinoderm Microtubule-Associated Protein-like 4 and Anaplastic Lymphoma Kinase
ERCC1 - excision repair cross-complementation group 1
ERK – extracellular signal pathway regulated kinase
ERMETIC - Study of the French National Cancer Institute platform
ETT TUKEB - Hungarian Scientific and Research Ethics Committee of the Medical Research Council
EUS - endoscopic ultrasonography
FDA – Food and Drug Administration
FFPE - formalin-fixed paraffin-embedded tissue
FISH - Fluorescence In Situ Hybridization

G - guanine
G12A - mutation results in an amino acid substitution in exon 2 at codon 12 in *KRAS*, from a glycine to an alanine
G12C - mutation results in an amino acid substitution in exon 2 at codon 12 in *KRAS*, from a glycine to a cysteine
G12D - mutation results in an amino acid substitution in exon 2 at codon 12 in *KRAS*, from a glycine to an aspartic acid
G12R - mutation results in an amino acid substitution in exon 2 at codon 12 in *KRAS*, from a glycine to an arginine
G12S - mutation results in an amino acid substitution in exon 2 at codon 12 in *KRAS*, from a glycine to a serine
G12V - mutation results in an amino acid substitution in exon 2 at codon 12 in *KRAS*, from a glycine to a valine
G13D - mutation results in an amino acid substitution in exon 2 at codon 13 in *KRAS*, from a glycine to an aspartic acid
G719x - exon 18 mutation, glycine change in the amino acid position 719
GAP – GTPase activating proteins
GDP - guanosine diphosphate
GEF – guanine nucleotide exchange factor
GPCR - G protein–coupled receptor
GTP - guanosine triphosphate
GTPase - guanosine triphosphatase
H&E - H&E Hematoxylin-Eosin
HER – human epidermal growth factor receptor
HR - hazard ratios
HRAS - Harvey rat sarcoma viral oncogene homolog
HRM - high resolution melting analysis
IGF – insulin-like growth factor
INSIGHT - ImplementatioN of perSonalized medicine In NSCLC in Central Europe: EGFR testing, Histopathology, and clinical feaTures observational study
IPASS - Iressa Pan-Asia Study
JBR.10 - Institute of Canada Cancer Therapeutics Group, JBR.10 was the North American Intergroup phase III trial of adjuvant cisplatin plus vinorelbine
JNK - C-Jun N-terminal kinase, belong to the mitogen-activated protein kinase family
KRAS - Kirsten rat sarcoma viral oncogene homolog
L - leucine
L858R - classic point mutation confers sensitivity to EGFR-TKIs and results in an amino acid substitution in exon 21 at position 858 in EGFR, from a leucine (L) to arginine (R)
L861Q - rare sensitizing mutation, and results in an amino acid substitution in exon 21 at position 861 in EGFR, from a leucine to glutamine
LeuArgGluAla motifs - "classic" microdeletions in *EGFR* gene confers sensitivity to EGFR-TKIs (exon 19 microdeletions at the amino acid position of 746–750)
LUX-Lung 2 - a phase II trial of the second-generation covalent TKI inhibitor afatinib

M - methionine
mABs - monoclonal antibodies
MAP – mitogen-activated protein kinase
MAP2KI - mitogen-activated protein kinase kinase 1
MAPK - mitogen-activated protein kinase
MEK - mitogen-activating protein kinase-kinase
MEK1 - mitogene activated protein kinase 1,
MET - mesenchymal-epithelial transition factor,
MIA - minimal invasive adenocarcinoma
MLPA - multiple ligation probe amplification
mTOR - mammalian target of rapamycin
MUT - mutation
NCCN - National Comprehensive Cancer Network
NGS - Next Generation Sequencing
NOS - otherwise specified
NRAS - neuroblastoma rat sarcoma viral oncogene homolog
NSCLC- non-small cell lung cancer
OD - optical density
ORR - overall response rate
OS - overall survival
P - Phosphorus
P value - probability value
p70S6k -70 kd S6 protein kinases
PCR – polymerase chain reaction
PCR-RFLP - polymerase chain reaction restriction fragment length polymorphism
PD - progressive disease
PD-1 - programmed cell death protein 1, that belongs to the immunoglobulin superfamily and is expressed on T cells
PDG / PDGFR – platelet derived growth factor / platelet derived growth factor receptor
PD-L-1 - programmed death-ligand 1
PFS - progression free survival
PI3K - phosphatidyl inositide 3-kinase
PIK3CA - phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3R1 - phosphoinositide-3-kinase regulatory subunit 1
PLCγ - phospholipase C-gamma
PR - partial response
PSA - prostate specific antigen
PTEN - phosphatase and tensin homolog
Q - glutamine
qRT-PCR – quantitative real-time PCR
R - arginine
Ral - Ras-related protein Ral-A

RAS - rat sarcoma viral oncogene homolog
RasGAPs - GTPase activating proteins
RECIST 1.1 - Response Evaluation Criteria in Solid Tumors
RFLP – restriction fragment length polymorphism
RNA - ribonucleic acid
ROS1 - homolog of the chicken c-ros, proto-oncogene, receptor tyrosine kinase, protein kinase ROS is an enzyme that in humans is encoded by the ROS1 gene
RR - response rate
RT-PCR - reverse transcription polymerase chain reaction
S - serine
S768I - point mutation results in an amino acid substitution in exon 20 at position 768 in EGFR, from a serine to threonine
SCLC- small cell lung cancer
SD - stable disease
SH-2 - Src Homology 2
STAT - signal transducers and activators of transcription pathways
T - timine
T790M - mutation results in an amino acid substitution at position 790 in EGFR, from a threonine to a methionine
Thr - threonine
TKI - tyrosine kinase inhibitor
TNM - Tumor Node Metastasis
TRIBUTE - Tarceva Responses in Conjunction with Paclitaxel and Carboplatin
TTNB - transthoracic needle biopsy
TTP - time to progression
UICC - Union for International Cancer Control (7th edition)
US - United States of America
V600E - BRAF mutation at amino acid position number 600 on the B-Raf protein, the normal valine is replaced by glutamic acid
VEGF / VEGFR – vascular endothelial growth factor / vascular endothelial growth factor receptor
VUSs - variants of unknown significance
WCLC - World Conference on Lung Cancer
WHO - World Health Organization
WT - wild-type
ZA – zoledronic acid

1. INTRODUCTION

1.1. Epidemiology

1.1.1. Global cancer trends

Worldwide, the most commonly occurring cancers are lung, prostate, breast, and colorectal [1]. Prostate, lung, and colorectal cancers will account for approximately one-half of all cases among males. Prostate cancer alone, accounts for about 25% of new diagnoses. The three most commonly diagnosed cancers among females are breast, pulmonary, and colorectal, and are responsible for 50% of all cases among females.

The increase in cancer incidence among males from 1970s to the 1990s attributed to a spike in prostate cancer mainly due to the increased discovery of asymptomatic disease through prostate specific antigen (PSA) testing. Effective therapies, like transurethral prostatectomy, were able to decrease cancer mortality and extend the patients survival [2]. The growth in female cancer incidence during the 1980s reflects the rise in breast and lung cancer cases driven by the tobacco epidemic, the changes in female reproductive patterns, and the detection of asymptomatic disease [3].

There are major worldwide variations in regional lung cancer incidence. Interestingly, an enormous geographic variation was described for lung cancer when compared to other malignancies [4]. Geographically, in Central and Eastern Europe and North America, males have the highest yearly lung cancer incidence rates (65.7 and 61.2 per 100,000; respectively). Among females, the highest rates were reported from North America and Northern Europe (35.6 and 21.3 per 100,000; respectively). State-specific lung cancer incidence rates are available for the United States of America (US). It was shown that Kentucky has the highest smoking prevalence with 3.5 times higher lung cancer incidence than those in Utah which has the lowest smoking prevalence [1].

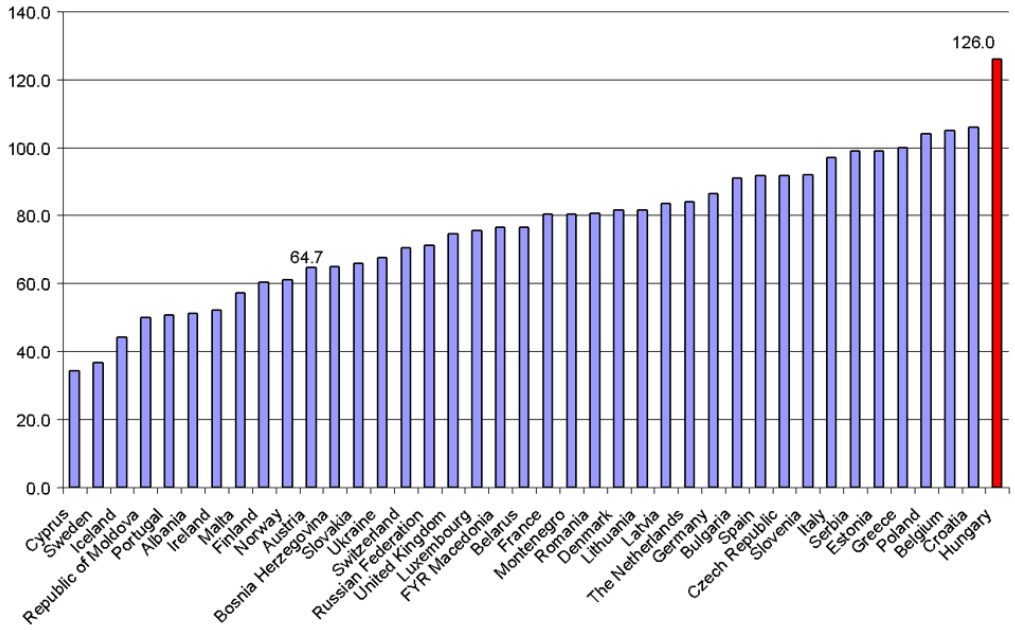
In contrast to the steady increase in overall survival (OS) for most malignancies, advances have been slow among patients with lung and pancreatic cancers. The 5-year relative survival is currently 18% and 7%, respectively.

These low rates are partly due to the aggressive biology of the tumor, the limited number of efficient therapeutic options, and more than 50% of the patients are diagnosed at advanced-stage for which the 5-year survival is 4% and 2%; respectively. The 5-year relative survival rate differs according to tumor stage, from 52% to 24% to 4% for local, regional, and advanced or metastatic stage disease; respectively. Higher tumor stage, older age, and male gender associates with worse prognosis [1, 5]. Early detection using computed tomography (CT) demonstrated to reduce lung cancer mortality by 16% among smokers and increased the 3 years survival rate of lung adenocarcinoma patients with 26.2% when compared to chest radiography [5]. In the US, the lung cancer mortality trends and cancer death rate rose during most of the 20th century as a result of the changes in smoking habits. Since the 1990s, however, there has been decline in the number of all cancer deaths due to decreased tobacco use, advances in early detection and cancer prevention.

1.1.2. Lung cancer in Hungary

Hungarians, geographically located in East-Central Europe at the Carpathian Basin, have one of the highest incidence rates for lung cancer in Europe (***Figure 1***). In particular, Hungarian males have the world's highest lung cancer mortality rate [6]. It should be noted that in the past two decades as smoking has decreased in the US, mortality has consequently declined [1]. However, there are no similar changes either in smoking habits or mortality in Asia, Europe, and Hungary [6, 7]. According to the literature, 10% of patients with lung cancer in the US are never-smokers; in Asia, more than 30% fall into this category [8]. A survey by Ostoros and co-workers conducted in 2012 demonstrated that 15% of lung cancer patients treated at the National Korányi Institute of Pulmonology, Hungary were never smokers, 33% former smokers, and 52% were found to be current smokers at the time of diagnosis [7].

A



B

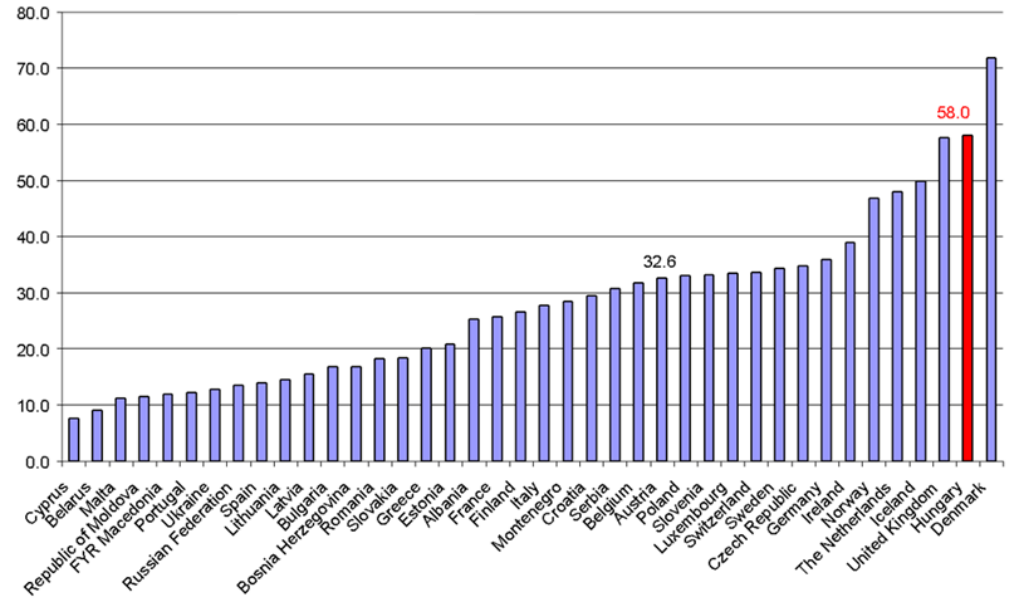


Figure 1. Crude rate of male (A) and female (B) lung cancer incidence in Europe [6].

World Health Organization (WHO) reported Hungary as a hot spot region in lung cancer. The highest incidence is in the Southeast (***Figure 2***).

Figure 2. Lung cancer incidence in 2013 in Hungary. The highest lung cancer incidence was reported from the Southeast of Hungary [7].

Currently, more men than women suffer from lung cancer in Hungary. However, the gender gap continues to narrow and is expected to eventually close in 2026 (***Figure 3***).

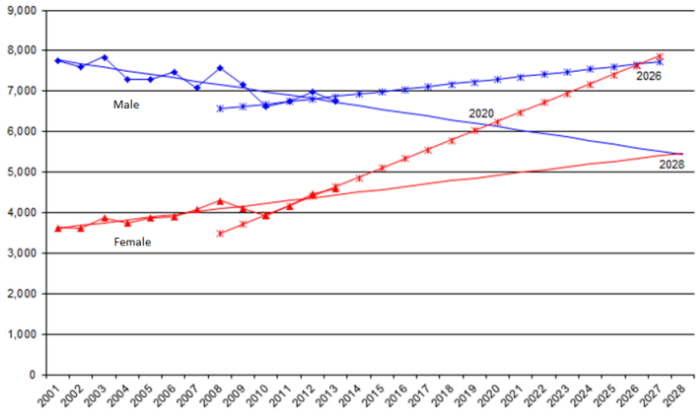


Figure 3. Expected incidence of lung cancer in the next two decades in Hungary. The extrapolated incidence of lung cancer among females (red) is going to reach those of the males (blue) by 2028. When we take into account the mean incidence during the last four years, the number of newly diagnosed lung cancer cases among females is expected to reach those of the males earlier, in 2026 (Modified after István H. Gaudi, Hungarian National Cancer Register).

In contrast to Hungary, lung cancer incidence rates in the US began to decline in the 1980s among men and in the late 1990s in women as a result of reductions in smoking prevalence that began decades earlier. Contemporary differences in lung cancer incidence patterns between men and women reflect historical differences in tobacco use. Women quit smoking in large numbers decades later than men [1].

The Hungarian Central Statistical Office published a report covering changes in the structure of causes of death in Hungary between 2000 and 2012. Mortality due to cancer was found to increase, accounting for every four deaths overall. The ratio of deaths attributable to cancer definitively increased from 2000 to 2012, from 26.5% and 24.6% in 2000 to 28.9% and 27.5% (males and females, respectively) in 2012. During the same period, the ratio of deaths associated with the respiratory system increased from 3.8% to 5.2%. In 2014, the lung cancer prevalence in Central Hungary increased compared to the previous years. The absolute number of lung cancer patients was higher than 21,000 [7].

Currently, 40% of lung cancer patients are female, however, in the 1990s it was only 19%. In Budapest, the male-female ratio is close to one. There are no differences among age specific distribution of lung cancer incidence. Lung cancer mainly occurs among elderly people, with more patients 80 years old or older. Incidence in patients under 40 years is rare. According to disease stage at diagnosis, significant changes have not occurred in Hungary. The increase in prevalence has stopped.

1.2. Molecular background

1.2.1. Oncogenic driver mutations in lung adenocarcinoma

The term lung cancer represents a rather heterogeneous group of diseases, including conditions of varying etiology and molecular background.

Lung cancer is divided based on prognosis and therapeutic possibilities into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 85% of all lung cancer cases and consists of two main types: squamous cell carcinoma (25%) and non-squamous carcinoma (including adenocarcinoma (45%), large-cell carcinoma (1%), and other types (4%)) [7, 9].

Adenocarcinoma is the most frequently occurring histological subtype among non-smokers. As a result of the development of molecular classification, treatment should no longer rest on histological categorization based on the most recent National Comprehensive Cancer Network (NCCN) guideline (version 2. 2016) and might be replaced by a classification based on driver molecular alterations in the future [10]. In addition, based on the new adenocarcinoma classification, use of the term bronchioloalveolar carcinoma (BAC) is no longer recommended. New terms are currently suggested such as in situ pulmonary adenocarcinoma (AIS), minimal invasive adenocarcinoma (MIA), invasive adenocarcinoma, and variants of invasive adenocarcionoma.

In addition, the new molecular diagnostic and therapeutic possibilities have dramatically altered the classification and management of NSCLC. Identification of the so called ”driver” oncogenic mutations plays a decisive role in the development of different tumor types and will open the way to targeted biological therapies. Patient responses to classical therapeutic regimens within a molecular subgroup may also vary [11-13].

Today, the close link between lung cancer and smoking is a well-established fact. According to global statistics, 80% of male lung cancer patients are current or former smoker; among female patients, the ratio is at least 50% [8]. The new molecular biological methods have shown that there are basic differences in genetic alterations between smokers and non-smokers, a fact that may also influence therapeutic outcomes [14]. *Epidermal growth factor receptor (EGFR)* and *Kirsten rat sarcoma viral oncogene homolog (KRAS)* gene mutations occur almost in 50% of the Caucasian lung adenocarcinoma patient population. Most recent data from the MyCancerGenome database reported an increasing number of additional gene mutations associated with lung adenocarcinoma (***Table 1***) [15]. Nevertheless, the determination of "driver" oncogenic mutations (e.g. KRAS, EGFR, anaplastic lymphoma kinase (ALK), and homolog of the chicken c-ros proto-oncogene 1 (ROS1)) that play a crucial role in tumor development is pivotal to identify targets for therapy.

For the time being, three gene mutations play a key role in the treatment of NSCLC: the activating (sensitizing) mutation of the *EGFR* gene and the *Echinoderm Microtubule-Associated Protein-like 4 and ALK fusion gene (EML4-ALK),* and *ROS1* fusions [16-18]. While the demonstration of an *EGFR* mutation is required in order to prescribe epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) treatment for lung adenocarcinoma patients, the most recent NCCN guideline version 2.2016 did not report comprehensive requirements for the mutation testing methods. In addition, based on preclinical data, amino acid-specific subtype driver mutations may have influence on the therapeutic efficacy and indicate that the simple definition of *KRAS*-mutated tumor is not enough (without the definition of the specific mutation present) to identify patients with a different probability of responding to therapy in both lung and colon cancers [12, 19].

Overall, translational research is often instrumental in the identification of new therapeutic targets.

Demonstrating an overall survival benefit is rather challenging for such an aggressive malignancy, especially in locally advanced or metastatic stage cancer. We are in an era where there is an urgent, unmet need to increase the number of lung cancer patients who can benefit from efficient therapies.

Oncogenic ALK fusion was first described in lung cancer in 2007 [18]. ALK is a tyrosine kinase receptor, a transmembrane protein, with Src Homology 2 (SH-2) and phospholipase C-gamma (PLCγ) binding sites at its C terminal end. EML4 is its most frequent fusion partner. It activates the phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3)/ protein kinase B (PKB), also known as AKT/ mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK)/ mitogen-activating protein kinase-kinase (MEK)/ extracellular signal pathway regulated kinase (ERK) and signal transducers and activators of transcription (STAT) pathways. It occurs in 3-7% of NSCLCs. *EML4-ALK* is a translocation, a mutually excluding mutation with *EGFR* and *KRAS* [15]. It can be detected by reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry or fluorescence in situ hybridization (FISH). Currently in Hungary, it is part of the routine diagnostic procedure in NSCLC (when *KRAS* and *EGFR* mutations are not present).

ROS1 is a receptor tyrosine kinase of the insulin receptor family. Downstream signalization of ROS1 fusions via G protein–coupled receptor (GPCR) pathways lead to cell growth and proliferation and apoptosis inhibition. It occurs in 1-2% of NSCLC cases, especially in young never-smoker lung adenocarcinoma patients [16]. *ROS1* is mutually exclusive with other driver oncogenes, such as *EGFR, KRAS* and *ALK* positive tumors. It is sensitive to crizotinib and can be detected by immunohistochemistry and FISH (with a 15% cut-off value for positivity).

V-Raf (viral rapidly accelerated fibrosarcoma) murine sarcoma viral oncogene homolog B1 (B-Raf) is a serine/threonine protein kinase, a member of the Raf kinase family plays a role in regulating the mitogen-activated protein kinase (MAP kinase)/ERKs signaling pathway, which regulates cell division and, differentiation. *BRAF* mutations are frequently found in former/current smokers. It occurs in 1-3% of NSCLC cases, more frequently in lung adenocarcinomas [20]. *BRAF* mutations are found to be non-overlapping mutations with other oncogenic drivers. The most common mutation is at

amino acid position number 600 on the B-Raf protein, the normal valine is replaced by glutamic acid "V600E" on exon 15. It can be detected by sequencing or high resolution melting analysis (HRM).

Mesenchymal-epithelial transition factor (MET) is a receptor tyrosine kinase, also known as hepatocyte growth factor receptor, localized on chromosome 7. Downstream pathways of MET regulates cell survival (PI3K-AKT-mTOR), and other pathways involved in cell proliferation (RAS-RAF-MEK-ERK). It can be detected by quantitative real-time PCR (qRT-PCR). MET amplification can be found in 2-4% of NSCLCs, and its presence is associated with poor prognosis. MET amplification may occur in 20% of EGFR-TKI resistant tumors. In addition, MET exon 14 mutations were identified in 3% of nonsquamous NSCLCs may become important therapeutic targets in NSCLC [21].

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA) and its important downstream signaling protein is AKT. It is tested by RT-PCR-based assay or sequencing. It is found in both never-smokers and smokers and occur in 3.9% of squamous cell carcinomas and 2.7% of adenocarcinomas. *PIK3CA* mutations have been shown to confer resistance to EGFR-TKI therapy.

MEK1 mutation occurs in 1% of NSCLCs [22]. It is detected by sequencing. *MEK1* mutations confer sensitivity to MEK inhibitors. Mutations are predominantly transversions, and associated with smoking. Furthermore, they can be inhibited by trametinib, a MEK inhibitor drug.

Table 1. Oncogenic driver mutations in lung adenocarcinoma [15].

Gene	Alteration	Frequency in NSCLC
AKT1	Mutation	1%
ALK	Rearrangement	3–7%
BRAF	Mutation	1–3%
DDR2	Mutation	~4%
EGFR	Mutation	10–35%
FGFR1	Amplification	20%
HER2	Mutation	2–4%
KRAS	Mutation	15–25%
MEK1	Mutation	1%
MET[a]	Amplification, mutation	2–4%
NRAS	Mutation	1%
PIK3CA	Mutation	1–3%
PTEN	Mutation	4–8%
RET	Rearrangement	1%
ROS1[a]	Rearrangement	1%

Key of colors:
Drugs approved in NSCLC.
Drugs approved in NSCLC but for other molecular subtype.
Drugs approved in other cancer.
Drugs in clinical development.

1.2.2. Oncogenic functions of EGFR

EGFR was first described as a biomarker in lung cancer in 2004 [23]. EGFR, one of the growth factor transmembrane receptors (***Figure 4***), is a well-known oncogene: EGFR consists of three domains: a ligand-binding extracellular domain, a lipophilic transmembrane, and a cytoplasmic tyrosine kinase domain. After bonding with the ligand, the receptors are homo- or heterodimerized, which leads to autophosphorylation, followed by the activation of downstream signaling pathways.

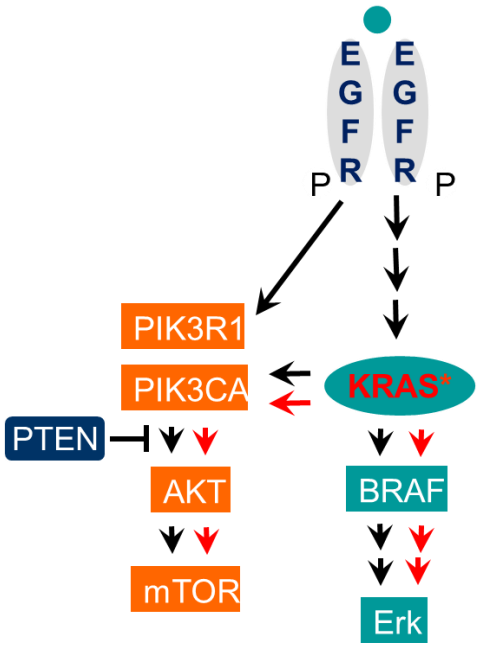


Figure 4. The EGFR signaling pathway. Upon activation, EGFR form dimers and activate the downstream effector, which induces activation of the BRAF/MEK/ERK (green). The PI3K/PTEN/AKT/mTOR pathways and as alters transcription by the activation of STAT. Mutations of the EGFR, KRAS, BRAF, and PIK3CA can lead to constitutive and ligand independent activation of the EGFR downstream signaling. Importantly, some of the above-mentioned molecules are currently targetable (Table 1). [24]. Activation of EGFR or KRAS downstream signalization indicated by black and red arrow, respectively.
BRAF, v-raf (viral rapidly accelerated fibrosarcoma) murine sarcoma viral oncogene homolog B1; MEK, mitogen-activating protein kinase-kinase; ERK, extracellular signal pathway regulated kinase; P, Phosphorus; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PI3K, phosphatidylinositide 3-kinase; PIK3CA, phosphatidylinositol-4,5-

bisphosphate 3-kinase catalytic subunit alpha; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; mTOR, mammalian target of rapamycin.

EGFR plays a physiological role in the growth, metabolic and cell regulation processes that are regulated by EGF, transforming growth factor (TGF) and several other ligands. These phosphorylated residues serve as docking sites for a variety of second messengers that can lead to downstream signaling activation. The downstream effectors such as RAS/RAF/MEK/ERK, phosphatidyl inositide 3-kinase (PI3K)/AKT and Janus kinase (JAK)/ STAT pathways, transduce signals in the nucleus, modulating gene expression, leading to DNA synthesis, and driving cell cycle progression. The above-mentioned proteins drive migration, adhesion, and proliferation. Of note, many downstream pathways participate in significant 'cross-talk' as well (***Figure 4***).
Somatic mutations of the *EGFR* gene can cause change in structure of the EGFR tyrosine kinase domain encoded by exon 18-21 of the *EGFR* gene. This genetic alteration can lead to constitutive and ligand independent activation of the EGFR downstream signaling.

1.2.3. Oncogenic functions of KRAS

The relevance of Ras in cancer was discovered three decades ago when it was proved that mutations in *RAS* had transforming activities of sarcoma-inducing retroviruses in rats. *KRAS* gene is a member of the *RAS* family that also includes of three genes: *H (Harvey), K (Kirsten) and N (Neuroblastoma)* [25]. Malignant activation of *KRAS* was first described in lung cancer in 1984 [26].
RAS is a proto-oncogene that is a central regulator of the growth factor receptor tyrosine kinase signaling cascades. Ras (p21Ras) is a guanosine triphosphatase (GTPase) protein activated protein family. Ras proteins have a key role in the connection of growth factor receptor tyrosine kinase signals and downstream intracellular signaling cascades (***Figure 4***). Receptor tyrosine kinase signals can activate guanine nucleotide exchange factors (RasGEFs), inducing the exchange of guanosine diphosphate (GDP) bound to Ras to guanosine triphosphate (GTP).

This change leads to RasGTP, which can activate important Ras effector pathways, such as B-raf/MEK/Erk, PI3K/Akt/mTOR and Ras-related protein Ral-A/c-Jun N-terminal kinas (C-Jun N-terminal kinase, belonging to the mitogen-activated protein kinase family (JNK)/ Ras-related protein (Ral) signals. During signal transfer RasGTP hydrolyzes into RasGDP, an inactive form, and needs another RasGEF signal to be reactivated. An important feedback is existing including RasGTPase activating proteins (RasGAPs), which can turn Ras into its inactive form (GTP-GDP change without signaling). These pathways have a key role in cell signaling affecting changes in cell cycle, proliferation, migration and apoptotic cell death.

RAS mutations are present in about 30% of all human cancers [27-29]. Massive clinical data shows that *KRAS* and *EGFR* mutations are mutually exclusive (with rare exceptions) [30]. The different KRAS isoforms are mainly present in different cancer types. *KRAS* mutations have been reported in NSCLC, colorectal carcinoma, pancreatic, endometrial, cervical and biliary tract cancers. *HRAS* mutations are most prevalent in bladder carcinomas, whereas *NRAS* mutations are most prevalent in melanomas [31].

KRAS mutations most frequently occur in lung adenocarcinoma, but are less frequently observed in squamous cell carcinoma of the lung. These mutations occur in exon 2 at codons 12, 13, and 61, and result in constitutive activation of Ras. Mutations have been observed predominantly (95%) at codon 12 and rarely in codon 13 or 61. *KRAS* mutations were identified predominantly among smokers and smoking history is considered as the most relevant risk factor for developing *KRAS* mutant lung adenocarcinoma.

These mutations impair the intrinsic GTPase activity of Ras and confer resistance to GTPase activators, thereby causing Ras to accumulate in its active guanosine triphosphate (GTP)-bound state, sustaining the activation of Ras signaling [32].

Despite the increased activity of the signaling pathway, the mutation alone causes loss of an enzyme function (RasGTPase). Since it is more difficult to recover the loss of a function than inhibit the effect of a mutation involving gain of function, attempts at targeting KRAS have remained unsuccessful for a long time. The efficacy of EGFR-TKI agents are also affected by *KRAS*. Although *KRAS* mutations were described as a negative predictive factor for EGFR-TKI therapy in several publications, the *EGFR* molecular diagnostic test is the recommended test in patient selection for TKI administration [27].

Transversion, in molecular biology, refers to the substitution of a (two ring) purine for a (one ring) pyrimidine or conversely in DNA. In genetics, a transition is a point mutation that changes a purine nucleotide to another purine (adenine (A) ↔ guanine (G)) or a pyrimidine nucleotide to another pyrimidine (cytosine (C) ↔ thymine (T)).

The frequency and spectrum of *KRAS* subtype mutations differs among cancer types. For example, in colorectal cancer, the most frequent change is a G to A transition (92% of mutations); however, in NSCLC in current smoker patients, the most common *KRAS* mutation is a G to T transversion. At codon 12 and/or codon 13 a G to A transition results in *KRAS* proteins in which the wild-type (WT) glycine residue is replaced by an aspartate, a valine or a cysteine. In NSCLC, the most common *KRAS* amino acid replacements in exon 2 at codon 12 and/or codon 13 are (47% of tumors) cysteine (Cys), (24%) valine (Val), (15%) aspartate (Asp), and (7%) alanine (Ala) [33].

Different *KRAS* oncogene substitutions have different effects on downstream signaling (***Figure 5***). NSCLC cell lines with G12D *KRAS* mutation had activated PI3-kinase and mitogen-activated protein/extracellular signal-regulated kinase kinase signaling, whereas those with G12C *KRAS* mutation or G12C *KRAS* mutation had decreased Akt activation, and increased Ral signaling.

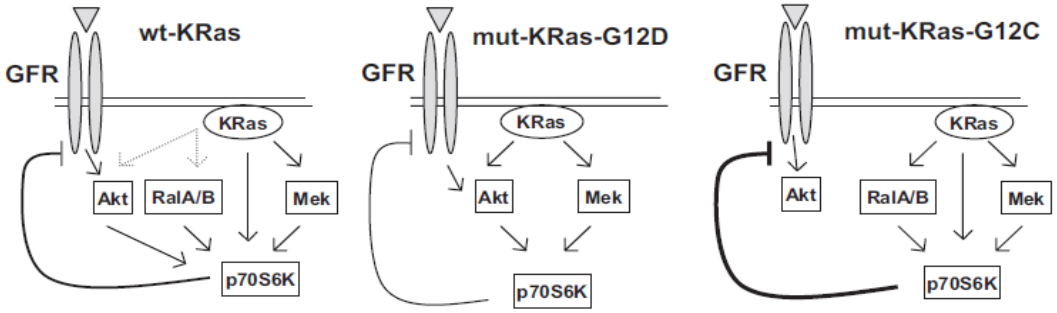


Figure 5. Effect of different *KRAS* oncogene substitutions on downstream signaling [33]. Activation of Akt, RalA, RalB and Mek signaling. Downstream signal transduction is represented by arrows. P70S6K is activated and exerts feedback inhibition on (E)GFR - mediated activation of Akt. *KRAS* G12D show weak inhibition, wild-type *KRAS* associated with moderate, and there is strong inhibition by *KRAS* G12C mutation.

p70S6k -70 kd S6 protein kinases; Ral, Ras-related protein Ral-A

In NSCLC, different amino acid-specific subtype *KRAS* mutations lead to a different downstream signaling and drug sensitivity. At this time, targeted therapy against mutant *KRAS* is unavailable. Nevertheless, KRAS mutation status has important clinical implications due to the urgent need of strategies for the treatment of *KRAS*-expressing tumors.

1.2.4. Molecular diagnostic methods in lung adenocarcinoma

In the past 20 years, there has been a significant advance in molecular diagnostics of solid tumors. With the application of molecular analysis, driver oncogenic aberrations can be identified, therapeutic targets defined, and their prognostic and predictive role can be recognized. In the near future, we might be able to identify an increasing number of tumor-associated genes and as a result, define a more extensive genetic map and biological features of a specific tumor.

Of genomic aberrations identifiable by molecular analysis, such as *KRAS, EGFR, EML4-ALK, MET, MEK, ROS1*, and *PI3K*, all have oncogenic relevance in thoracic oncology, with targeted therapies available or under development. Indeed, targeted therapy is an integral part of the current routine clinical practice in NSCLC.

As earlier mentioned, the demonstration of *EGFR* mutation is required to prescribe first line EGFR-TKI treatment for lung adenocarcinoma patients. There are no comprehensive requirements for the sample preparation, molecular diagnostic procedures, or the type of *EGFR* mutations that needs to be identified. However, very recent NCCN guideline version 2.2016 contain emerging and more extensive related data.

Molecular diagnostic methods can be divided into "screening" and "targeting," also known as "hot spot," techniques [34, 35]. Screening methods are capable of detecting all types of mutation (known or unknown (unpublished) mutations), but have a lower sensitivity and often require more time and experience than targeting techniques that analyze a specific target section of the gene. While this latter approach has a higher sensitivity and is quicker, it is often more expensive.

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) a routine diagnostic technique suitable for detecting variations in homologous DNA sequences, such as *KRAS* mutations [36]. By the use of the RFLP method, homologous DNA molecule variations can be detected. It splits the DNA strand along a specific nucleotide sequence. The steps include polymerase chain reaction (PCR), digestion and gel electrophoresis. The DNA sequence is separated according to its length by gel electrophoresis. The number of DNA fragments after splitting depends on the number of sequences identified by the enzyme, while their size depends on their distance separated by gel electrophoresis. After dividing the restriction, fragments are tested by Southern-blot hydrolysation. RFLP (mutation) appears if the insertion, deletion or point mutation on the examined DNA strand destroys an existing restriction site or creates a new one. In Hungary, this is the initial step in the sequential diagnostic algorithm of lung adenocarcinomas.

Allele specific PCR technique focuses on polymorphic mutant segments. Cytological samples taken by the endobronchial ultrasound (EBUS) and endoscopic ultrasonography (EUS) techniques had good correlation with allele specific PCR, when compared to results based on histological samples [37].

During the use of high resolution melting analysis (HRM), the required DNA segment is amplified by PCR. The amplified segment is called amplicon. The amplicon is heated at 50-90 °C. Once it has reached its melting point, it divides into two. It is then stained with fluorescent, intercalating dye. Decrease (change) in fluorescence is measured and melting curves are plotted. Accordingly, mutation changes the shape of the curve.

Mass spectrometry, a system enables sensitive and rapid somatic mutation profiling. Of note, rare and potentially targetable mutations can be detected with this method. Sequenom's OncoCarta Panel v3.0 is a set of pre-validated assays for cost-effective, efficient mutation testing. Fresh, frozen, or formalin fixed, paraffin embedded tissues (FFPE) can be used to analyze 105 mutations with only 480 ng DNA per FFPE.

Due to its high level of specificity, Sanger sequencing is considered a gold standard [38]. During DNA sequencing, the base sequence of the nucleotides of a DNA segment is defined on an amplicon (a specific gene segment).

Through sequencing, each nucleotide of a specific gene segment is identified. Identification not only includes the presence of mutation but also its exact nucleotide sequence can be recognized and used for analysis. It should be pointed out that even rare mutations, or variants of unknown significance (VUSs), can be detected by this method. These mutations can become new targets in the future.

Pyrosequencing is a screening method, with DNA polymerase activity is measured using chemiluminescent staining. That it relies on the detection of pyrophosphate release on nucleotide incorporation. In addition to classic EGFR mutations, it can identify certain rare sensitizing EGFR mutations in lung adenocarcinomas.

Next Generation Sequencing (NGS) is a method where DNA strands are tested one by one, in a quantity of several millions. NGS is quicker and less expensive than Sanger sequencing. Previous study showed that, NGS data was 100% identical with direct sequencing [39]. It is capable of recognizing many genes, that can be analyzed easily. Such as previously characterized changes (mutations and benign SNPs), simple substitution mutations to complex deletion and insertion mutations, regions of a gene typically not tested for mutations, like deep intronic and promoter mutations, can also be detected, but also may result in sequencing errors. "Targeted resequencing" or validation by direct sequencing can be a solution.

FISH uses probes of various colors that are hybridized for the tested gene and bind to the chromosomes due to high-level sequence complementarity. For *ALK* translocation to be present in NSCLC, 15% of the cells must be positive .

All in all, point mutations and minor deletions can be detected by PCR-RFLP, allele specific PCR, HRM, Sanger sequencing, pyrosequencing, and NGS, gene rearrangement can be analysed with FISH, NGS, and PCR, microsatellite instability can beidentified by fragment analysis, and major deletions can be recognized by multiple ligation probe amplification (MLPA).

When selecting a molecular diagnostic test, the type and tumor content of the sample, equipment and experience of the testing laboratory, as well as the type of the mutation, (i.e. frequent or rare, to be identified), should be taken into consideration [34]. When comparing molecular biological techniques, detection and identification of mutations, as well as sensitivity are to be considered.

While mutations can be detected on a specific probe region, as is the case with PCR-RFLP and allele specific PCR, or on a specific amplicon as done by Sanger sequencing, HRM and NGS are capable of recognizing more extensive range of mutations. Sanger sequencing with 99.9% accuracy is the "gold standard" for clinical research sequencing. Sanger sequencing, pyrosequencing and NGS can identify exact nucleotide sequence of mutations. In contrast, PCR-RFLP and allele specific PCR are incapable of determining the precise nucleotide sequence of mutations.

HRM also cannot identify the exact type of mutation and must be followed by sequencing. Sanger sequencing has a sensitivity of 20%, pyrosequencing and HRM 5-10%, allele specific PCR 5%, while PCR-RFLP and NGS have shown a sensitivity of 2% [40].

While pyrosequencing can only be used to focus in a targeted way (e.g. on classic activating mutations), direct sequencing enables testing for rare mutations. Determined by mutation specific techniques, such as pyrosequencing or COBAS (Roche), classic mutations are reported in the literature accounting for 90% of all existing mutations.

Overall, in the treatment of NSCLC routine testing for *ALK* gene rearrangements and *EGFR* mutations are recommended. The NCCN guideline recommends molecular test for nonsquamous NSCLC or NSCLC not otherwise specified (NOS). In rare cases, mixed histology including squamous cell cancer can possess *ALK* rearrangements or sensitizing *EGFR* mutations. Accordingly, in squamous cell lung cancer, molecular test for *EGFR* and *ALK* can have relevance in never-smokers, patients with small biopsy samples and if mixed histology was reported.

1.3. Current therapeutic regimens in lung cancer

1.3.1. Chemotherapy regimens for advanced or metastatic disease in non-small cell lung cancer

Current NCCN guideline (version 2.2016) recommends selection for systemic chemotherapy based on the tumor histology. Platinum-based chemotherapy increase survival and quality of life. Platinum-based combinations show 25%-35% response rate

(RR) and an 8-10 month expected median OS. Patients presenting with Eastern Cooperative Oncology Group performance status (ECOG PS) 3-4 do not benefit from cytotoxic treatment.

In the first line setting, platinum-based chemotherapy together with pemetrexed is superior in nonsquamous when compared to gemcitabine combination which is superior in squamous cell histology. For patients with squamous cell carcinoma, cisplatin/gemcitabine or cisplatin/vinorelbine or carboplatin/paclitaxel is recommended. Pemetrexed or bevacizumab is not recommended for squamous cell carcinoma. Doublet agents like cisplatin or pemetrexed are usually administered to patients with nonsquamous, *EGFR* or *ALK* negative NSCLC. The addition of bevacizumab to platinum/paclitaxel chemotherapy is the category 1 recommendation for selected cases and recommended to patients with brain metastases as well.

In the second-line setting immune checkpoint inhibitors are preferred agents based on improved response, survival and less adverse events among advanced nonsquamous NSCLC patients that had progressed during or after platinum-based chemotherapy. Nivolumab, a programmed death 1 (PD-1) immune-checkpoint-inhibitor improves survival when compared with docetaxel [41].

Pemetrexed monotherapy show similar efficacy when compared to docetaxel. A randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced NSCLC previously treated with platinum-based doublets showed that docetaxel is superior to vinorelbine [42]. Ramucirumab (human IgG1 monoclonal antibody that targets the extracellular domain of VEGFR-2) and docetaxel are superior to docetaxel alone [43]. Pemetrexed monotherapy has similar efficacy, but with significantly fewer side effects compared to docetaxel alone in adenocarcinoma (and large cell) histology.

Currently, we do not have established predictive biomarkers for chemotherapy. In NSCLC the excision repair cross-complementation group 1 (ERCC1) molecule was shown to be a predictive biomarker for cisplatin therapy. Although, the possible accessible ERCC1 antibodies did not specifically recognize the unique functional ERCC1 isoform. Consequently, its comprehensive clinical utility is not yet established [44].

Although several groups investigated *KRAS* mutations in NSCLC patients treated with chemotherapy, the predictive power of *KRAS* mutational status as a marker for chemosensitivity in NSCLC also remains controversial [28, 45, 46].

1.3.2. Molecular targeted therapy in lung cancer

The development of new targeted therapies involves not only the invention of novel therapies for well-known target molecules, but also the identification of new indications for already established biomarkers and targets [47]. Finding new indications is not always obvious, because the same treatment can have opposite effect on cancer cells. Amino acid-specific subtype mutations can alter the protein structure and may lead to drug sensitivity or resistance to a specific targeted therapy. Receptors encoded by molecular alterations can result in amino acid changes or can be silent without any change in the protein structure. The mutations with amino acid changes can be divided into two categories: conservative (amino acid replacement with similar biochemical features) or non-conservative (different protein structure). Understanding these mechanisms can help in development of new targets and therapies. Furthermore, combined treatments can lead to a more efficient usage of known targeted therapies and to successful treatment of resistant cases.

Crizotinib targets ALK, ROS1, and MET [48]. Ceritinib acts on ALK and insulin-like growth factor 1 receptor (IGF-1). All of these drugs can be orally administered. Crizotinib is category 1 recommendation based on a phase III clinical trial for patients with locally advanced or metastatic *ALK* positive NSCLC ECOG PS 0-4. A phase II clinical trial showed dramatic 80% reponse rate (RR) to patients that previously progressed on chemotherapy. Ceritinib is Food and Drug Administration (FDA) approved for metastatic patients who did not tolerate or progressed on crizotinib [49].

Very recently (December 11, 2015) through accelerated process, FDA approved alectinib, a second generation agent for the treatment of advanced *ALK*-positive NSCLC. According to the approval, this medication intended after progression or intolerance to crizotinib and can be administered orally. Based on the results of single arm studies the RR was found to be 38% to 44% and the median PFS was 7.5-11.2 months. Alectinib showed excellent RR (66%) and median PFS of 9.1 months, especially for patients with brain metastasis [50].

Bevacizumab is a recombinant monoclonal antibody that blocks vascular endothelial growth factor receptor (VEGFR, VEGF-A) and administered intravenously. The combination of paclitaxel and carboplatin with bevacizumab showed a significant survival benefit (vs. chemotherapy alone, 14.2 vs. 10.0 months, respectively) with the risk of increased treatment-related deaths. There were 15 treatment-related deaths in the paclitaxel and carboplatin plus bevacizumab subgroup, including 5 from pulmonary hemorrhage [51].

Agents targeting BRAF, RET, MET, ROS1, human epidermal growth factor (HER) are in clinical trials or under development. BRAF V600E mutant tumors can be inhibited by dabrafenib, vemurafenib and dabrafenib plus trametinib. MEK1 is targeted by trametinib. *HER2* mutations positive tumors can be inhibited by trastuzumab or afatinib (category 2B recommendations).

In December 2015, European Medicines Agency (EMA) approved ramucirumab, in combination with docetaxel. The drug is indicated for the treatment of locally advanced or metastatic NSCLC after progression to platinum-based chemotherapy. Additionally, EMA recommended granting a conditional marketing authorization (product that accomplishes an unmet medical necessity) for osimertinib, an irreversible EGFR-TKI, intended for the treatment of locally advanced NSCLC with sensitizing *EGFR* mutations and a specific TKI-resistance mutation (T790M). This indication is approved under accelerated approval based on RR and duration of response.

Despite the always-emerging identification of relatively rare occurring oncogenes and the increasing approval of targeted therapies, *KRAS* - the most frequently occurring oncogene - currently is not targetable. Furthermore, guidelines lack comprehensive information on the predictive role of *KRAS*.

Nevertheless, the routine clinical use of KRAS gene testing is not widely established, KRAS mutations are considered to be a negative predictor for EGFR-TKI therapy and mutually exclusive with other oncogenic driver mutations [46]. However, the latter statement also has some ambiguity [52, 53] and thus EGFR mutational status analysis is currently the preferred test in this setting [27, 54]. Monoclonal antibodies (mABs) against EGFR as monotherapy or in combination with chemotherapy confirmed efficacy only in KRAS WT colorectal cancer [54, 55]. The relevance of EGFR mAbs in NSCLC was not

confirmed. However, necitumumab, a recombinant IgG1 human monoclonal antibody designed to bind and block the ligand binding site of EGFR is under development.
There is an ongoing phase II study of paclitaxel and carboplatin chemotherapy plus necitumumab (LY3012211) in the first-line treatment of patients with stage IV squamous NSCLC [56]). Also, a clear association between KRAS mutations in NSCLC and efficacy of anti-EGFR mABs has not been demonstrated [57, 58].

1.3.3. EGFR targeted therapy in lung cancer

The identification of somatic mutations in *EGFR* as a clinically applicable biomarker was first published in 2004 [17]. In lung cancer, oncogenic mutations of the *EGFR* are the most frequent and biologically targetable molecular alterations. To date, most of the drugs introduced in therapy are TKIs, which can be administered after *EGFR*, and once *KRAS* mutation analyses have been performed. A well known fact is that "classic" point mutation confers sensitivity to EGFR-TKIs and results in an amino acid substitution in exon 21 at position 858 in EGFR, from a L to arginine (R) (L858R) and exon 19 microdeletions (LeuArgGluAla motifs at the amino acid position of 746–750) can serve as positive predictive biomarkers for EGFR-TKI therapy [23]. These mutations are referred to as classic sensitizing *EGFR* mutations. The presence of *EGFR* activating mutations are responsible for increased oncogenic activation, and the binding of tyrosine kinase inhibitors to the same region. In addition to the classical activating mutations, several other gene mutations occurring in the exon 18-21 of the *EGFR* gene (rare EGFR mutations) may have a potential role as oncogenic activating mutation.
Oral TKIs that inhibit the EGFR tyrosine kinase domain prevent the dimerization and therefore inhibits the downstream signaling. Furthermore, there are many rare mutations in the *EGFR* gene in NSCLC and the clinical relevance and the correlation with response to TKI that remain unclear [59, 60]. According to the NCCN guidelines, there is a significant association between certain rare *EGFR* mutations and sensitivity to EGFR-TKIs.

Specifically, the exon 18 mutation glycine change at the amino acid position 719 (G719x) and the exon 20 point mutation resulting in an amino acid substitution at position 768 in EGFR, from a serine (S) to threonine (T) (S768I) and L861Q, which results in an amino acid substitution in exon 21 at position 861 in EGFR, from a leucine (L) to glutamine (Q) demonstrated sensitivity to EGFR-TKIs.

It should also be noted that there are known *EGFR* mutations which are responsible for the presence or development of resistance to TKI therapy. The *EGFR* mutation results in an amino acid substitution at position 790 in EGFR, from a threonine to a methionine (M) (T790M) and exon 20 insertion mutations are considered to be resistance mutations [61].

Classic *EGFR* mutations occur almost exclusively in adenocarcinomas. Their incidence, however, greatly varies in different populations, showing the highest frequency among East-Asian non-smoker females. There is an inverse relationship between smoking status and frequency of classic *EGFR* mutations [8]. However, the association between smoking and the frequency of rare *EGFR* mutations remains unclear. The epidemiology and clinical relevance of rare *EGFR* mutations are also not yet clearly established.

Erlotinib, gefitinib, afatinib, and osimertinib are inhibitors of EGFR. Since 2004, FDA approved erlotinib for patients with locally advanced or metastatic NSCLC with sensitizing *EGFR* mutations. The Iressa Pan-Asia Study (IPASS) study compared erlotinib to paclitaxel/carboplatin and showed increased PFS and RR for the erlotinib arm [62]. The OS was the same for both arms; however, the quality of life was increased in the erlotinib arm. Afatinib is also approved for first line therapy or subsequent lines of therapy based on data showing efficacy in patients who have progressed after first line chemotherapy [63, 64].

The actual NCCN guideline recommends *EGFR* mutation testing in patients with advanced nonsquamous NSCLC. However, no specific mutation test is recommended. Of note, there is an emerging number of mutations associated with increased response to EGFR-TKIs recommending molecular testing.

It should also be noted that there are known *EGFR* mutations which are responsible for the presence or development of resistance to TKI therapy.

The factors responsible for EGFR-TKI resistance may include the presence of *EGFR* resistance mutations (*EGFR* T790M and exon 20 insertion mutations), *MET* amplification and mutation, as well as mutations of other genes involved in signal transmission, such as *BRAF* or *PI3K*. It is interesting to note that the incidence of *EGFR* T790M mutation may be as high as 60% before EGFR-TKI administration using the mutant enriched PCR technique [65]; by means of direct sequencing, however, a rate of 0-1% was reported [66]. Of note, *MET* amplification may occur in 20% of EGFR-TKI resistant tumors.

1.3.4. Immunotherapy in lung cancer

Immunotherapy can demonstrate antitumor efficacy thru upregulating cancer specific immune systems. Immune checkpoints limit or block immune response, tumors often use this mechanism to reduce anti-tumor immune responses. Negative co-stimulation can downregulate the immune system [67].

Programmed cell death protein 1 (PD-1), member of the immunoglobulin superfamily is expressed on T cells. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), is a type of membrane protein found on activated antigen presenting cells (B7) [68]. These particles are examples of co-inhibitory checkpoint molecules. Nivolumab is one example of an immunomodulator thru blocking ligand activation of the PD-1 receptor on stimulated (activated) T cells [41]. Ipilimumab is a monoclonal antibody that can enhance the tumor specific immune response thru CTLA-4, a receptor that decreases the immune response.

1.3.5. Prognostic biomarkers in lung adenocarcinoma

The aforementioned dismal outcome of lung cancer underlines the urgent needs for prognostic and predictive biomarkers. A prognostic biomarker is indicative of OS unrelated to the therapy administered. It reflects the tumor biology and aggressiveness.

Several clinicopathological variables were identified as prognosticators for lung adenocarcinoma. Good prognostic factors include early-stage disease at diagnosis, good performance status (ECOG PS <=2), no significant weight loss (<5%) and female gender. Smoking is an important prognosticator, as several studies have demonstrated that never-smokers have improved OS [69, 70].

Classic *EGFR* mutant cases significantly more frequent among never-smokers than rare *EGFR* mutant ones. Thus, it is likely that the increased survival is owing to the overall better performance and the lack of smoking related co-morbidities [69-72]. The positive prognostic value of the *EGFR* mutation has been challenged recently [73].

Furthermore, it remains unclear whether classic *EGFR* mutation (exon 19del or exon 21 (L858R)) itself confers a more benign behavior or the increased RR to TKI therapy translates to better prognosis.

In resected stage I-II NSCLC, published data revealed that *KRAS* mutations were linked with a negative prognosis [74, 75]. In 1991, RAS mutation was a negative prognostic factor also in advanced-stage NSCLC, irrespective of the treatment intent [76]. A meta-analysis has shown that KRAS mutations are associated with poor prognosis. In the participating studies varying molecular methods were performed, patients with different tumor stages were enrolled, and diverse treatments were administered. This latter finding has limited clinical utility [27].

2. OBJECTIVES

A number of clinicopathological factors influences the incidence and clinical consequence of oncogenic driver mutations. Therefore, in this thesis, we aimed to investigate the epidemiology and clinical relevance of subtype-specific *KRAS* and *EGFR* mutations in lung adenocarcinoma.

1. In advanced-stage lung adenocarcinoma, the clinical significance of amino acid substitution-specific *KRAS* mutational status in terms of tumor progression after chemotherapy and OS has not yet been clearly established. Therefore, in order to better understand the influence of *KRAS* mutations in this setting, we analyzed a large cohort of Caucasian patients with unresected stage III-IV lung adenocarcinoma who were treated with platinum-based chemotherapy.

2. Furthermore, in advanced-stage lung adenocarcinoma, the clinical significance of rare *EGFR* mutations has not yet been clearly established [77, 78]. Therefore, we analyzed a large cohort of Caucasian patients with known *KRAS* and *EGFR* mutational status to compare the epidemiology and clinical consequence of rare and classic *EGFR* mutations.

3. While *KRAS* mutation is a negative predictive marker for EGFR tyrosine kinase inhibitor therapy, there is limited data available regarding the influence of *KRAS* mutation on the organ specificity of lung adenocarcinoma dissemination. Therefore, the aim of our study was to investigate the metastatic site-specific prognostic value of *KRAS* mutation in lung adenocarcinoma patients.

3. METHODS (Materials and methods)

3.1. Ethics Statement

The retrospective studies and all treatments were conducted in accordance with the current National Comprehensive Cancer Network guidelines, based on the ethical standards prescribed by the Helsinki Declaration of the World Medical Association and with the approval of the national level ethics committee that included a waiver for the retrospective studies (52614-4/2013/EKU). Informed consent was obtained from all patients that received TKI treatment or chemotherapy. Patients were de-identified following the clinical information collection. As a result, patients cannot be identified either directly or indirectly based on our datasets.

3.2. Study Population

Consecutive patients with cytologically or histologically confirmed, advanced lung adenocarcinoma evaluated at the National Koranyi Institute of Pulmonology and at the Department of Pulmonology, Semmelweis University between 2009 and 2013 were analyzed in these retrospective studies. Based on the inclusion criteria, we set up three patients cohort. In all study cohorts, the molecular analysis was performed for potential anti-EGFR-TKI therapy indication. Cohort #1 was dedicated to understand the clinical role of amino acid-specific subtype *KRAS* mutations in lung adenocarcinoma. The cohort #2 focused on the epidemiology and clinical relevance of rare *EGFR* mutations. The combined cohort investigated the site-specific variations in KRAS status according to metastatic sites.

All patients were Caucasians. Tumor Node Metastasis (TNM) staging of the tumor according to the Union for International Cancer Control (7th edition) [79], smoking status, ECOG PS, and age was evaluated at the time of diagnosis. For the purpose of clinicopathological characterization, the study population was divided into three smoking

categories: ’never-smokers’ including those who had smoked less than 100 cigarettes during their lives; ’former smokers’ including those who had smoked more than 100 cigarettes but had not smoked for at least a year; and ’current smokers’ for those who still smoked. Passive smoking was not taken into account.
The pre-therapeutic tissue samples (cytology or histology), were obtained by surgery, transthoracic needle biopsy (TTNB), bronchoscopy or CT-guided biopsy. The diagnosis was established according to the WHO criteria.

3.2.1. EGFR mutations (cohort #1)

In this cohort, patients had pathologically confirmed lung (recurrent stage was not included) adenocarcinoma treated between January 2010 and March 2013. All patients undergoing *EGFR* and/or *KRAS* mutation identification tests required for potential anti-*EGFR* therapy were included in the analysis. *KRAS* and/or *EGFR* mutation status had been defined in 814 and 602 patients, respectively. Retrospective clinical data (performance status, smoking history, and tumor stage) was available for 646 patients and their correlations with mutational status were analyzed for epidemiological purpose. In the advanced-stage lung adenocarcinoma patient cohort full clinical follow-up was available for 419 patients. Clinical follow-up was closed on November 1, 2013.

3.2.2. KRAS mutation subtype and platinum based first line therapy (cohort #2)

In this retrospective analysis, 505 patients with unresectable stage III or IV lung adenocarcinoma were included who underwent first-line platinum-based (cisplatin or carboplatin) doublet regimen between January 2009 and May 2012. All patients were subject to *KRAS* mutation testing and they were (re)staged using the seventh edition of the TNM classification [80]. Clinical follow-up was closed on February 1, 2013.

3.2.3. Metastatic pattern and KRAS mutations (combined cohort)

In our retrospective, single center study, 903 lung adenocarcinoma patients with *KRAS* mutation analyses were included. At the time of diagnosis, 500 patients had metastatic disease. These cases were analyzed for the potential association between *KRAS* status and metastatic site and clinical outcome. Due to the strong association with better prognosis and different therapeutic regimens, patients with known *EGFR* mutations were excluded from the study. Clinical follow-up was closed on May 30, 2015.

3.3. Mutation Analysis

For the current study, all mutational analyses were performed at the 2nd Department of Pathology and at the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University as previously described in [81]. Briefly, regions of tumor samples embedded in paraffin blocks containing the highest concentrations of tumor cells were macro-dissected [82]. DNA was extracted using the MasterPure™ DNA Purification Kit according to the manufacturer's instructions. As in the introduction already mentioned, in Hungary KRAS testing is performed at first to exclude KRAS mutant cases from EGFR analysis as part of a diagnostic algorithm elaborated to reduce costs and to optimize testing and therapeutic efficiency. This screening strategy also allows the analysis of large number of cases for KRAS mutations.

3.3.1. KRAS mutation analysis

KRAS mutations were evaluated by microcapillary-based RFLP analysis characterized by 5% mutant tumor cell content sensitivity as previously described in [81]. The base-pair substitution in the mutant samples were verified and determined by sequencing on the ABI 3130 Genetic Analyzer System (Life Technologies, Carlsbad, CA) with the BigDye® Terminator v1.1 Kit.

3.3.2. EGFR mutation analysis

In the *EGFR* mutation identification procedure, PCR amplification of the *EGFR* gene specific to exons 18, 19, 21 in 459 patients (76%) and exons 18, 19, 20, 21 in 143 (24%) cases was the initial step, followed by bidirectional Sanger sequencing of PCR products. Sensitivity of this molecular test is nearly 20% (able to detect mutations in specimens with at least 20% cancer cell content); its specificity is aproximately 100% [34]. In other cases (n=7) the TheraScreen: EGFR29 Mutation Kit (DxS Ltd., UK) was used to identify activating mutations relevant to EGFR-TKI therapy. This technique has a sensitivity of approximately 1% (able to detect mutations in specimens with at least 1% cancer cell content) and a specificity of 100% [34].

3.4. Treatment and follow-up

Treatment efficacy was assessed from contrast-enhanced CT performed at baseline before treatment initiation and then every subsequent 3 months afterwards. Therapy responses were categorized as per Response Evaluation Criteria in Solid Tumors (RECIST 1.1) best response along the treatment period (stable disease [SD], partial response [PR], and complete response [CR]) or progressive disease [PD], was evaluated in the retrospective analysis. Overall response rate (ORR) was calculated as the number of patients with a best response of CR or PR divided by the total number of patients in each (treatment) group.

3.4.1. EGFR-TKI treatment

Indications for EGFR-TKI therapy were: advanced lung adenocarcinoma patients with ECOG PS 0-3 received in 2nd and 3rd lines erlotinib (orally at a daily dose of 150 mg) with *KRAS* wild-type tumor from January 2010, meanwhile 1st line gefitinib (250 mg/day

orally) was available from March 2012 for patients with activating *EGFR* mutation. Treatments were administered until disease progression or intolerable toxic effect. Adverse events were assessed according to the Institute Common Terminology Criteria (3.0) [83]. The study and all treatments were conducted in accordance with NCCN guidelines. The patients were asked to return to the hospital every month for review including vital signs, performance status, and adverse events. Only patients with initial ECOG PS 0-2 and complete clinical follow-up were included in the retrospective analysis investigating the clinical relevance of rare *EGFR* mutations. Patients were defined as primary resistant if they showed no response to gefitinib at any time (only patients received TKI treatment for at least 1 months were included) and if progression occurred within the first 3 months of treatment.

3.4.2. Platinum-based chemotherapy

Patients with initial ECOG PS 0 or 1 and complete clinical follow-up were included. For the calculation of PFS date of the first chemotherapy was used. Clinical follow-up in the subtype-specific *KRAS* cohort was closed on February 1, 2013. Only patients with complete documentation of treatment were included. According to our inclusion criteria, in cohort #1 all patients were treated with a first-line platinum-based doublet regimen (unresectable stage III patients received chemotherapy in combination with radiotherapy). Patients were treated with cisplatin or carboplatin. Platinum was most frequently given together with paclitaxel. Other combination drugs were gemcitabine, pemetrexed and docetaxel.

3.5. Statistical Methods

Overall survival was estimated from diagnosis for patients presenting with unresectable advanced-stage III/IV disease, until death or last available follow up. Progression free survival (PFS) was calculated from the date of initiation of treatment to the date of detection of PD or death. Categorical parameters of the patients with different mutational status were statistically analyzed by Chi-square test. Kaplan-Meier survival curves and two-sided Log-rank tests were used for univariate survival analyses of categorical impact factors.

The Cox proportional hazards model was used for uni- and multivariate survival analyses to detect the impact of both continuous and categorical factors and to calculate the hazard ratios (HR) and corresponding 95% confidence intervals (CI). P values are always given as two-sided and were considered statistically significant below 0.05. Metric data is always shown as median or mean and corresponding range or, in case of OS and PFS, as median and corresponding 95% CI. All statistical analyses were performed using the PASW Statistics 18.0 package (Predictive Analytics Software, SPSS Inc., Chicago, IL, USA).

3.6. In vitro experiments

3.6.1. Cell lines and culture conditions

The eight human NSCLC cell lines used in the experiments were obtained from American Type Culture Collection (ATCC). The H358 [84], CALU3 [85], and the A549 [86] cell lines were *KRAS* mutant. *EGFR* mutant cell lines were the H1975 [87] and H1650 (in the latter, additional PTEN loss was identified [87], BRAF mutants were the CRL 5885 [87], and CRL5922 (in the latter, additional NRAS mutation was found) [88] cell lines. The HCC78 cell line expresses the *ROS1* fusion [89].

Cell cultures were maintained in DMEM (Lonza, Switzerland; with 4500 mg/dm^3 glucose, pyruvate and L-glutamine) supplemented with 10% fetal calf serum (Lonza) and 1% penicillin-streptomycin-amphotericyn (Lonza) in tissue culture flasks in a humidified 5% CO_2 at 37°C.

3.6.2. Clonogenic assay

The antiproliferative effect of zoledronic acid (ZA) treatment was evaluated by clonogenic assay. Briefly, 1000 cells were seeded in six well plates and treated 1, 2, 8, and 32 μM ZA for 10 days. Fresh medium and ZA were supplied on each 3rd day. On the 10th day cells were fixed with trichloroacetic acid (10%) and stained for 15 min with Sulforhodamine B. Cells were washed 3 times with acetic acid 1% (vol/vol) to remove excess dye. The protein-bound dye was dissolved in 10 mM Tris. Optical density (OD) was determined at 570 nm by a microplate reader (EL800, BioTec Instruments, and Winooski, VT). Data shown as average of two independent experiments and effect of treatment is expressed relative to control.

4. RESULTS

4.1. Molecular epidemiology of driver oncogenic mutations in advanced lung adenocarcinoma

Patient cohorts and mutational analysis flow chart are shown in ***Figure 6.*** The molecular epidemiology and therapeutic consequences of driver oncogenic mutations were analyzed in each different patient cohorts.

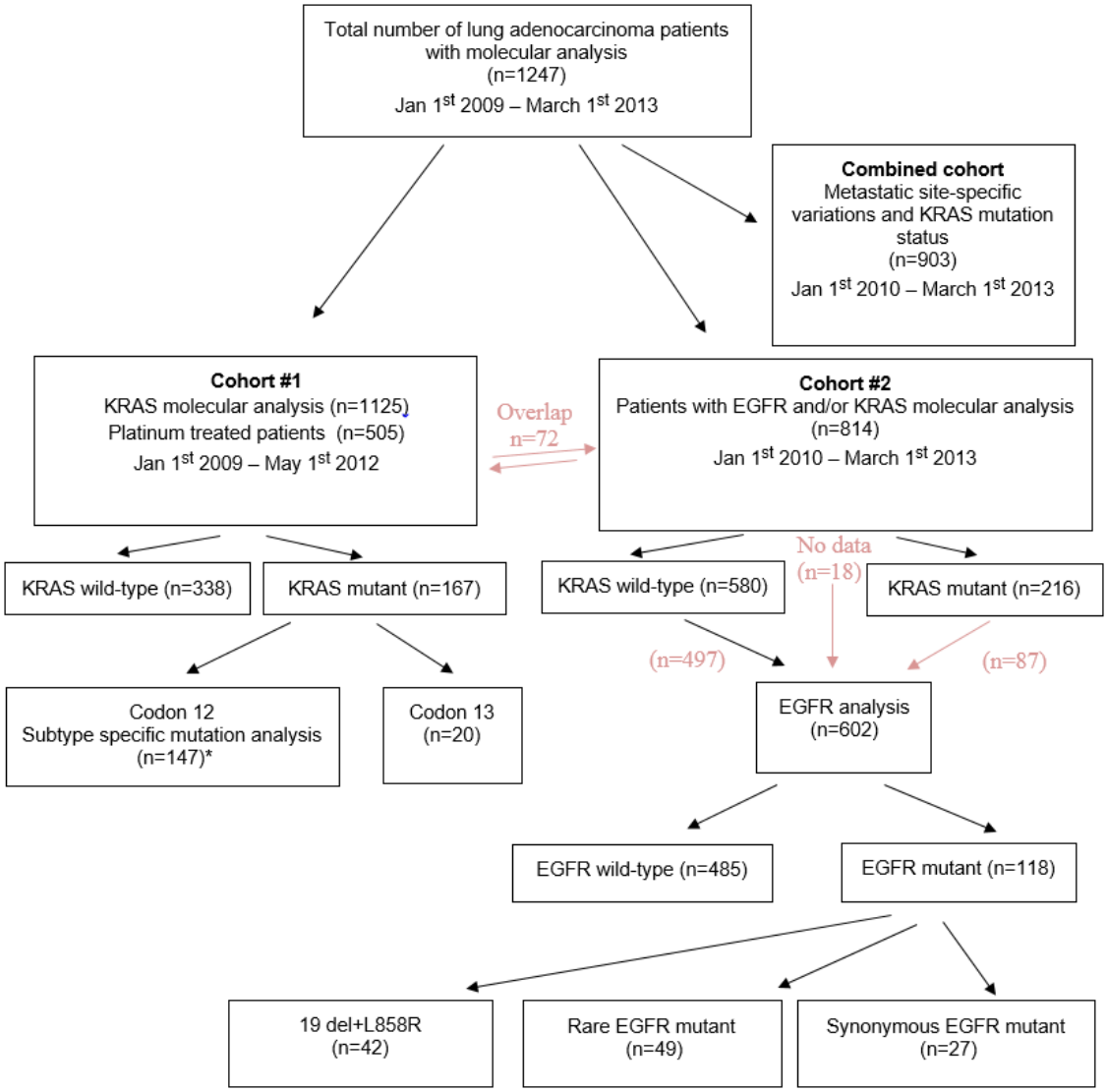


Figure 6. Patient cohorts and mutational analysis flow chart (n=1247 patients).

*In 11 *KRAS* codon 12 mutant cases the exact nucleotide change was not identifiable

4.1.1. Incidence of KRAS mutations

The molecular epidemiology of *KRAS* mutations according to the patient cohorts are shown in ***Table 2.*** In cohort #1, the total number of patients with *KRAS* mutational status available was 1125. Seven hundred and sixty four (68%) cases were identified as *KRAS* WT, 335 (30%) as *KRAS* codon 12 mutant and 26 (2%) as *KRAS* codon 13 mutant. The overall mutation rate was 32% (361 out of 1125). Thus 93% of the mutations occurred on codon 12 and 7% had a codon 13 mutation.

In cohort #2, we identified 580 patients as *KRAS* WT (73%) and 216 as *KRAS* mutant (27%). In 18 cases, no *KRAS* mutation analysis was performed, (***Figure 6.***).

In the combined cohort out of the 903 patients, 647 *KRAS* WT (72%) and 256 *KRAS*-mutant (28%) cases were identified.

Table 2. Molecular epidemiology of *KRAS* mutations.

	Cohort #1		**Cohort #2**	**Combined cohort**
	All patients with KRAS mutation analysis	*Platinum treated patients*		
Total number	1125	505	814 *	903
KRAS wild-type	764 (68%)	338 (67%)	580 (73%)	647 (72%)
KRAS mutation	361 (32%)	167 (33%)	216 (27%)	256 (28%)

**KRAS* analysis was not performed in 18 cases.

In cohort #1, based on our inclusion criteria (platinum-based chemotherapy with initial stage III or IV disease and ECOG PS of 0 or 1 and complete clinical follow-up), we enrolled 338 *KRAS* WT (67%), 147 codon 12 mutant (29%) and 20 codon 13 mutant (4%) patients (***Table 3***).

The number of the major KRAS subtypes in cohort #1 was 61 (39%) G12C, 29 (18%) G12V, 27 (17%) G12D, and 8 (5%) G12A. In 31 cases rare KRAS codon 12 and 13 subtype mutations were identified.

4.1.2. Incidence of EGFR mutations

The epidemiology of *EGFR* mutations was investigated in cohort #2. Ninety-one patient carried non-synonymous *EGFR* mutation out of the 814 cases (***Figure 6.***).
There were 42 (5%) classic *EGFR* mutant (4 patients with concomitant *KRAS* mutation), 49 (6%) rare *EGFR* mutant (non-classic mutation where amino acid change occurs) (including 3 patients with concomitant *KRAS* mutation) and 27 (3%) patients with synonymous (silent) *EGFR* mutations (non-classic mutations without amino acid change in *EGFR*) (including 9 patients with concomitant *KRAS* mutation) and 480 (59%) of the cases was classified as *KRAS*/*EGFR* double WT (***Figure 7***). Of note, in five patients, the G719X or L861Q rare sensitizing mutation was identified [90]. All rare and synonymous *EGFR* mutations are listed in ***Supplemental Table 1.*** Based on the Catalogue Of Somatic mutations in cancer (COSMIC) database, we found synonymous and rare *EGFR* gene mutations already published in lung cancer (N=33 mutations) or in malignancies of other organs (N=20 mutations) [91]. Additionally, 45 previously unpublished novel mutations were identified. T790M mutation was not detected in any patients. Interestingly, in 16 patients, 39 mutations were identified within a complex mutation pattern (at least two different *EGFR* mutations within a single sample).

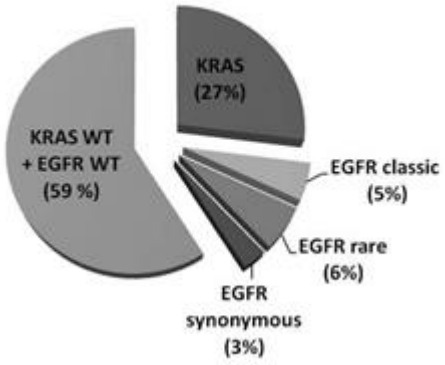


Figure 7. Distribution of *KRAS* and *EGFR* mutations in lung adenocarcinoma patients. Mutational status in the full cohort #2 (n=814). Wild-type: WT.

4.2. Clinicopathological characteristics of lung adenocarcinoma patients

In order to determine the clinical relevance of *KRAS* and *EGFR* mutations, we performed a comparative statistical analysis of mutational status and clinicopathological variables (summarized in ***Tables 3, 4, 5, 6, and 7***). The major clinicopathological characteristics could be collected in cohort #1 and in cohort #2 (505 and 645 patients, respectively) and are presented for the various mutational statuses in ***Tables 3 and 4.*** Similarly to the cohort #1, significant association between gender or ECOG PS and mutational status was not detected in cohort #2 (***Tables 4 and 5,*** and ***Figure 8A***).

In cohort #1, *KRAS* mutation was not significantly associated with age when patients were grouped as <55, 55-64 and 65≤ years (P=0.119). However, one-way analysis of variance (ANOVA) test with Tukey Multiple Comparison indicated a significant difference between the average ages of WT and *KRAS* codon 12 mutant patients (60.7 versus 58.8 years, respectively, P=0.032). In cohort #2 patients with *KRAS* mutations (mean age ±SD, 60±10.4 yrs.) were significantly younger than those with *EGFR*/*KRAS* double WT tumors (mean age ±SD, 64±9.7 years) or with classic *EGFR* mutations (mean age ±SD, 67±9.6 years) (P<0.001, ***Figure 8B***).

We found no significant association with major clinicopathological factors and amino acid-specific *KRAS* mutation subtypes.

Table 3. Correlation of clinicopathological features and *KRAS* mutational status in patients with advanced pulmonary adenocarcinoma in cohort #1 (n=505).

			KRAS status			
		No. of patients (%)	**WT (%)**	**KRAS12 (%)**	**KRAS13 (%)**	***P value***
	All patients	**505 (100%)**	**338 (67%)**	**147 (29%)**	**20 (4%)**	
Age (years) [a]	<55	109 (21.6%)	66 (19.5%)	35 (23.8%)	8 (40%)	0.119
	55-64	251 (49.7%)	166 (49.1%)	77 (52.4%)	8 (40%)	
	≥65	145 (28.7%)	106 (31.4%)	35 (23.8%)	4 (20%)	
Gender	Male	262 (51.9%)	186 (55%)	66 (44.9%)	10 (50%)	0.120
	Female	243 (48.1%)	152 (45%)	81 (55.1%)	10 (50%)	
ECOG PS	0	279 (55.2%)	190 (56.2%)	77 (52.4%)	12 (60%)	0.307
	1	226 (44.8%)	148 (43.8%)	70 (47.6%)	8 (40%)	
Smoking [b]	Never-smoker	63 (12.5%)	49 (14.5%)	13 (8.8%)	1 (5%)	0.059
	Ever-smoker	398 (78.8%)	249 (73.7%)	132 (89.8%)	17 (85%)	
Stage	III	167 (33.1%)	115 (34%)	47 (32%)	5 (25%)	0.668
	IV	338 (66.9%)	223 (66%)	100 (68%)	15 (75%)	

[a] Mean age was 60.1 years (range, 33-79; SD=8.04) for the entire patient population, 60.7 years (range, 33-79; SD=7.93) for the wild-type (WT) patients, 58.8 years (range, 39-78; SD=8.16) for the *KRAS* codon 12 mutant group, and 58.1 years (range, 47-73; SD=8.02) for the *KRAS* codon 13 mutant cohort. [b] In 44 cases, smoking status was not available; Data shown in parentheses are column percentages; ECOG PS, Eastern Cooperative Oncology Group performance status

Table 4. Characteristics of patients with major clinicopathological data available in cohort #2 (n=645).

		Total	**KRAS mutation**	**Classic EGFR mutation**	**Rare EGFR mutation**	**Synonymous EGFR mutation**	**KRAS and EGFR wild-type**	**P value**
Total		645* (100%)	112 (17%)	42 (7%)	49 (8%)	27 (4%)	415 (64%)	
Age (mean±SD)		64±10	60±10.4	67±9.6	64.2±9.2	63.2±9.8	64±9.7	**<0.001**
Gender	Male	303 (45%)	49 (44%)	19 (45%)	26 (53%)	11 (40%)	198 (48%)	0.780
	Female	342 (55%)	63 (56%)	23 (55%)	23 (47%)	16 (60%)	217 (52%)	
ECOG PS	0	325 (48%)	58 (52%)	25 (63%)	25 (51%)	15(58%)	202 (51%)	0.658
	≥1	300 (52%)	54 (48%)	15 (37%)	24 (49%)	11 (42%)	197 (49%)	
	Unknown data	20	1	2	0	1	16	
Smoking status	Never-smoker	118 (19%)	16 (14%)	20 (49%)	12 (24%)	4 (15%)	66 (17%)	**<0.001**
	Former smoker	198 (32%)	39 (35%)	13 (32%)	12 (24%)	9 (33%)	125 (32%)	
	Current smoker	302 (49%)	56 (51%)	8 (19%)	25 (52%)	14 (52%)	199 (51%)	
	Unknown data	27	1	1	0	0	25	
Tumor Stage	I-IIIA	275 (44%)	52 (47%)	19 (47%)	27 (56%)	20 (77%)	157 (39%)	**<0.001**
	IIIB-IV	351 (56%)	59 (53%)	21 (53%)	21 (44%)	6 (23%)	244 (61%)	
	Unknown data	19	1	2	1	1	14	

Data shown in parentheses are column percentages.
* In cohort #2, out of the total number of patients (n=814) with molecular analysis, clinicopathological data was available in 645 cases. EGFR molecular analysis was not done in 43 cases.
ECOG PS, Eastern Cooperative Oncology Group performance status

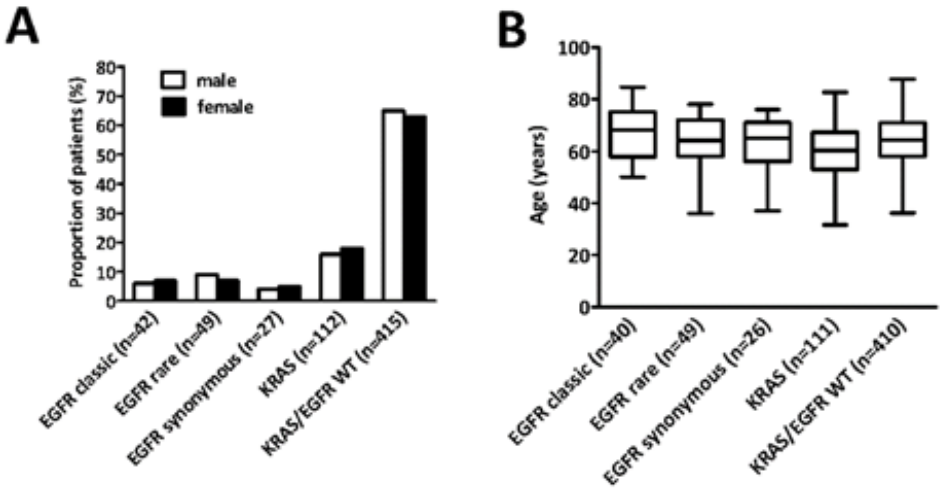


Figure 8. Epidemiology of *KRAS* and *EGFR* mutations in lung adenocarcinoma patients in cohort #2. (A) There was no significant association between mutational status and gender. (B) Patients with *KRAS* mutation were significantly younger than those with classic *EGFR* mutations or with *EGFR*/*KRAS* double wild-type (WT) tumors ($P<0.001$).

Table 5. Correlation of clinicopathological features, and *KRAS* codon 12 mutation subtypes in cohort #1 in patients with advanced pulmonary adenocarcinoma (n=136 [a]).

KRAS mutation [a]		**G12C (n=61)**	**G12V (n=29)**	**G12D (n=27)**	**Rare (n=19)**	***P value***
Age [b] (years)	<55	15 (24.6%)	6 (20.7%)	7 (25.9%)	4 (21.1%)	0.767
	55-64	35 (57.4%)	16 (55.2%)	13 (48.1%)	8 (42.1%)	
	≥65	11 (18%)	7 (24.1%)	7 (25.9%)	7 (36.8%)	
Gender	Male	28 (45.9%)	14 (48.3%)	13 (48.1%)	5 (26.3)	0.407
	Female	33 (54.1%)	15 (51.7%)	14 (51.9%)	14 (73.7%)	
Smoking	Never-smoker	3 (4.9%)	6 (20.7%)	1 (3.7%)	3 (15.8%)	0.055
	Ever-smoker	58 (95.1%)	23 (79.3%)	26 (96.3%)	16 (84.2%)	
ECOG PS	0	28 (45.9%)	16 (55.2%)	17 (63%)	10 (52.6%)	0.507
	1	33 (54.1%)	13 (44.8%)	10 (37%)	9 (47.4%)	
Stage	III	19 (31.1%)	8 (27.6%)	7 (25.9%)	8 (42.1%)	0.664
	IV	42 (68.9%)	21 (72.4%)	20 (74.1)	11 (57.9%)	

[a] Out of the 147 KRAS codon 12 mutant patients, in 11 *KRAS* codon 12 mutant cases the exact nucleotide change was not identifiable;
[b] Mean age was 58.8 years (range, 39-78; SD=8.16) for the entire *KRAS* codon 12 mutant group, 58.1 years (range, 39-76; SD=8.00) for the G12C patients, 59.5 years (range, 41-76; SD=8.14) for the G12V patients, 59.1 years (range, 39-75; SD=8.28) for the G12D patients, and 59.6 years (range, 40-78; SD=8.68) for patients with rare *KRAS* codon 12 mutations; Data shown in parentheses are column percentages; ECOG PS, Eastern Cooperative Oncology Group performance status.

4.2.1. Smoking and KRAS and EGFR mutation status

In cohort #1, smoking status and *KRAS* mutational status did not show a significant correlation (P=0.059; ***Figure 9A***). However, when *KRAS* mutant cases were combined (all *KRAS* WT patients vs. codon 12 plus codon 13 *KRAS* mutants; ***Table 3***) the tendency towards an increased frequency of *KRAS* mutations in ever-smoker patients reached a statistically significant level (P=0.0189; vs. never-smokers; Chi-square test). Accordingly, we found a significantly elevated risk for ever-smoker advanced lung adenocarcinoma patients to carry a *KRAS* mutation (RR=1.93; CI=1.1136-3.3512; P=0.0089) that translates to an almost two-fold risk of having a *KRAS* mutant tumor.

In cohort #2, *KRAS* mutant cases significantly associated with smoking status when compared to the double WT patient population (P<0.01; ***Figure 9B***). Classic *EGFR* mutation was significantly associated with never-smoker status when compared to all other mutational statuses (***Figure 9B***; P<0.0001). Next, we investigated the clinical relevance of subtype-specific *EGFR* and *KRAS* mutations. We found that rare *EGFR* mutations are associated with smoking (vs. classic *EGFR* mutations; ***Figure 9B***; P=0.0062).

Next, in cohort #1, we investigated the characteristics of patients with *KRAS* mutations in codon 12 and performed a statistical analysis on their association with amino acid-specific mutational status. Similar to the overall cohort, smoking status and specific *KRAS* codon 12 mutations showed an almost significant correlation (P=0.055, ***Table 3***). Therefore, the correlation of mutational status and smoking status was further analyzed (***Figure 9B***). Codon 12 *KRAS* mutations were significantly more frequent in current and/or former smokers than in never-smokers (P=0.032, ***Figure 9B***). Importantly, the amino acid-specific mutation subtype analysis identified G12V *KRAS* mutation as more frequent in never-smokers than among former and current (or ever) -smokers (***Figure 9C***)

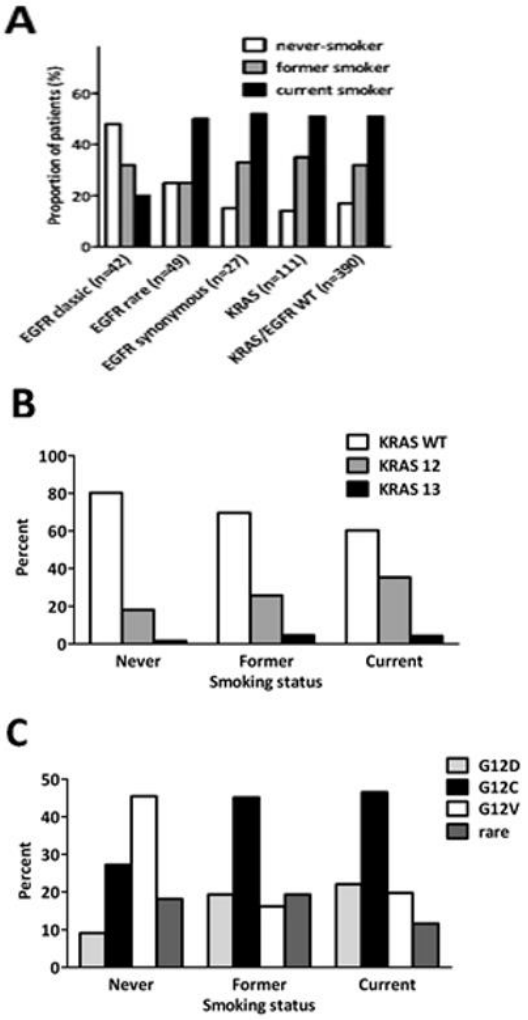


Figure 9. Distribution of patients according to driver oncogenic mutations and smoking status. (A) In cohort #2, rare *EGFR* mutations - in contrast to classic *EGFR* mutations - were significantly associated with smoking (P=0.0062). In cohort #1, (B) *KRAS* wild-type (WT), *KRAS* codon 12 and codon 13 mutants and (C) codon 12 subtype-specific KRAS mutants were analyzed. *KRAS* mutation is significantly more frequent among former or current than in never-smokers (P=0.032, Chi-square test). G12V *KRAS* mutation is more frequent in never-smokers.

4.2.2. Patient characteristics and metastatic pattern

Clinicopathological characteristics and *KRAS* mutational status of patients with different metastatic pattern are shown in ***Table 6*** and ***Table 7.*** Among the 903 consecutive lung adenocarcinoma patients identified, 256 (28%) were *KRAS* mutant and 647 (72%) were *KRAS* WT. Four hundred three patients presented with non-metastatic disease and 500 cases were metastatic at the time of diagnosis. We found 362 (72%) single-organ and 138 (28%) multiple-organ metastatic cases (***Table 6***). The most frequent metastatic sites included lung (45.6%), bone (26.2%), adrenal gland (17.4%), brain (16.8%), pleura (15.6%), and liver (11%).

We did not found significant differences in age in the metastatic (61.9±9.4) vs. non-metastatic (61.8±8.9) cohorts or patients with single-organ (62.33±9.3) vs. multiple-organ (60.8±9.7) metastases. Patients presented with only pleural spread (66.8±10.4) were significantly older than those with only lung (62±8.9), bone (60±10.7), adrenal (63.1±6.8), or brain (59.7±9.2) metastases (P=0.0024, P=0.0008, P=0.0132, P=0.002). Patients with brain metastases were significantly younger than those with lung spread (P=0.0094).

Only in the bone metastatic group we found a higher percentage of male patient when compared to females in adrenal, brain or lung group (56% vs. 49%, 43%, and 45%, respectively, P=0.0479). The proportion of ECOG PS 0-1 was similar in the different organ-specific metastatic subgroups. The proportion of never-smokers was significantly increased in patients with pleural metastases (27%) when compared to all other sites (12.2%, P=0.0018).

Table 6. Correlation of clinicopathological features, *KRAS* mutation status and metastatic pattern in the combined cohort at the time of diagnosis in patients with advanced pulmonary adenocarcinoma (n=903).

Metastatic pattern		**Multiple-organ**	**Single-organ**	**Non-metastatic**
Total		138	362	403
Age (mean±SD)		60.8±8.7	62.4±9.3	61.8±8.9
Gender	**Male**	64 (46%)	181 (50%)	190 (49%)
	Female	74 (54%)	181 (50%)	213 (51%)
ECOG PS	**0-1**	124 (92%)	335 (94%)	382 (96%)
	>1	11 (8%)	21 (6%)	15 (4%)
	Unknown data	3	6	6
Smoking status	**Never-smoker**	15 (12%)	52 (16%)	66 (17%)
	Former smoker	37 (30%)	104 (31%)	115 (30%)
	Current smoker	71 (58%)	179 (53%)	203 (53%)
	Unknown data	15	27	19
KRAS	**Wild-type**	94 (68%)	263 (73%)	290 (72%)
	Mutation	44 (32%)	99 (27%)	113 (28%)

Data shown in parentheses are column percentages.
Metastatic pattern was evaluated at the time of diagnosis. ECOG PS, Eastern Cooperative Oncology Group performance status.

Table 7. Clinicopathological features, *KRAS* mutation status and site specific metastatic pattern in the combined cohort at the time of diagnosis in patients with advanced lung adenocarcinoma (n=500*).

Metastatic site		**Lung**	**Bone**	**Adrenal**	**Brain**	**Pleura**	**Liver**
Total		228	131	87	84	78	55
Age (mean±SD)		62±9.0	60.7±10.2	61.1±9.6	59.2±9.3	64.5±10.5	62.2±9.9
Gender	**Male**	102 (45%)	74 (56%)	34 (39%)	36 (43%)	38 (49%)	26 (47%)
	Female	126 (55%)	57 (44%)	53 (61%)	48 (57%)	40 (51%)	29 (53%)
ECOG PS	**0-1**	218 (97%)	115 (91%)	75 (87%)	77 (93%)	71 (91%)	48 (91%)
	>1	7 (3%)	11 (9%)	11 (13%)	6 (7%)	7 (9%)	5 (9%)
	Unknown data	3	5	1	1	0	2
Smoking status	**Never-smoker**	29 (14%)	18 (16%)	7 (9%)	7 (9%)	20 (27%)	4 (8%)
	Former smoker	61 (29%)	32 (28%)	20 (25%)	25 (32%)	23 (32%)	20 (41%)
	Current smoker	117 (57%)	65 (58%)	52 (66%)	45 (58%)	30 (41%)	25 (51%)
	Unknown data	21	16	8	7	5	6
KRAS	**Wild-type**	148 (65%)	94 (72%)	58 (67%)	60 (71%)	65 (83%)	46 (84%)
	Mutation	80 (35%)	37 (28%)	29 (33%)	24 (29%)	13 (17%)	9 (16%)

Data shown in parentheses are column percentages.
*The number of site-specific metastatic cases included single and multiple organ metastatic patients at the time of diagnosis. ECOG PS, Eastern Cooperative Oncology Group performance status.

4.2.3. Metastatic site-specific variation of KRAS status

Metastatic site-specific variation of *KRAS* status is shown in ***Figure 10.*** There was no difference in the *KRAS* mutation incidence between the metastatic (28.6%) and non-metastatic cases (28%) (***Table 6***, ***Figure 10A***). Patients with multiple-organ metastases showed a non-significant increase in the percentage of *KRAS* mutation (vs single-organ spread 32% vs 27%, ***Table 7, Figure 10B***).

Importantly, patients with brain (29%), bone (28%) or adrenal gland (33%) metastases demonstrated similar *KRAS* mutation frequencies (***Figure 10C***). However, pulmonary metastatic cases demonstrated increased *KRAS* mutation frequency when compared to

those with extrapulmonary metastases (35% and 26.5%, P=0.0125, ***Figure 10C***). In contrast, pleural dissemination and liver metastasis associated with decreased *KRAS* mutation incidence (vs all other metastatic sites; 17% (P<0.001) and 16% (P=0.0023), respectively).

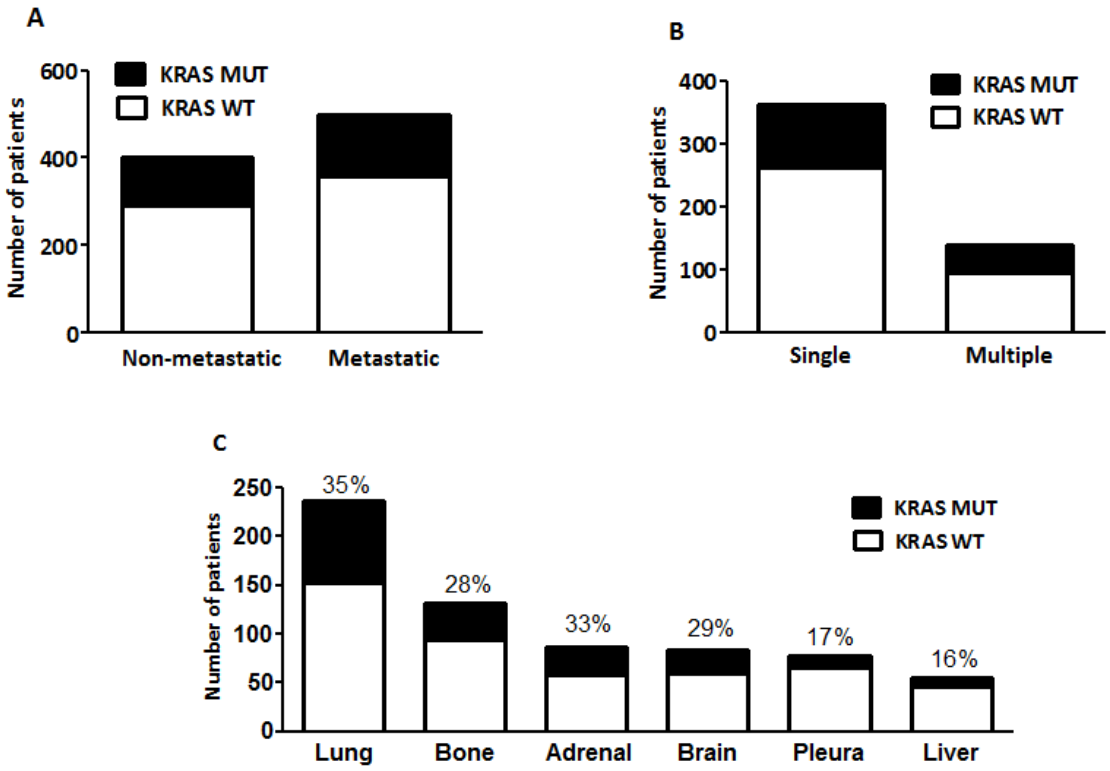


Figure 10. Metastatic site-specific variation of *KRAS* status. (A) Non-metastatic or metastatic patients (28% vs. 28.6%, ns, Chi-square test), and (B) patients with multiple-organ metastases showed a non-significant increase in the percentage of *KRAS* mutant cases (vs. single-organ spread, 32% vs. 27%). (C) In the organ-specific analysis, patients with brain (29%), bone (28%) or adrenal gland (33%) metastases demonstrated similar *KRAS* mutation frequencies. However, pulmonary metastatic cases demonstrated increased *KRAS* mutation frequency when compared to those with extrapulmonary metastases (35% vs. 26.5%, P=0.0125). In contrast, pleural dissemination and liver metastasis associated with decreased *KRAS* mutation incidence (17% (P<0.001) and 16% (P=0.0023), respectively). WT, wild-type; MUT, mutant; Single, single-organ; Multiple, multiple-organ metastasis.

4.3. Prognostic factors in advanced lung adenocarcinoma

4.3.1. Classical prognostic factors in advanced lung adenocarcinoma

Clinical follow-up including overall survival could be collected for all patients who met the inclusion criteria in cohort #1 (n=505), meanwhile in the advanced-stage cohort #2 (unresected stage IIIA, IIIB-IV) for 419 patients (***Supplemental Table 3***). Age, gender, ECOG PS, tumor stage, smoking status and mutational status were tested for discriminating power in predicting disease outcome. We found no significant difference in OS according to gender in cohort #1 (data not shown). However, in cohort #2, we found that male patients had significantly shorter OS (vs. females; HR 1.32; 95% CI, 1.04-1.66; P=0.0195, data not shown). In cohort #1, we observed that patients with ECOG 0 PS had significantly longer median OS than did ECOG PS 1 patients (P<0.001, log-rank test; ***Figure 11A***). Correspondingly, in cohort #2 patients with ECOG PS 0 had significantly longer median OS than those presenting with ECOG PS 1-2 (HR 2.07; 95% CI, 1.63-2.62; P<0.001; ***Figure 11B***). In cohort #1, we found no difference in OS in our patient cohort, according to smoking status (***Figure 11C***). In contrary, in cohort #2 we found significantly increased OS among never-smokers as compared to ever-smoker patients (HR, 0.666; 95% CI, 0.497-0.892; P=0.0063; ***Figure 11D***). We also found that patients in cohort #1 with stage III tumors had significantly longer OS than did patients with a stage IV tumor (23 vs. 11 months, P<0.001, log-rank test, ***Figure 11E)***. Stage IIIB or IV lung adenocarcinoma patients had significantly shorter OS than those with unresected stage IIIA (HR 0.637; 95% CI, 0.478-0.850; P=0.002; ***Figure 11F***). We found no significant difference in OS between stages IIIB or IV patients (data not shown).

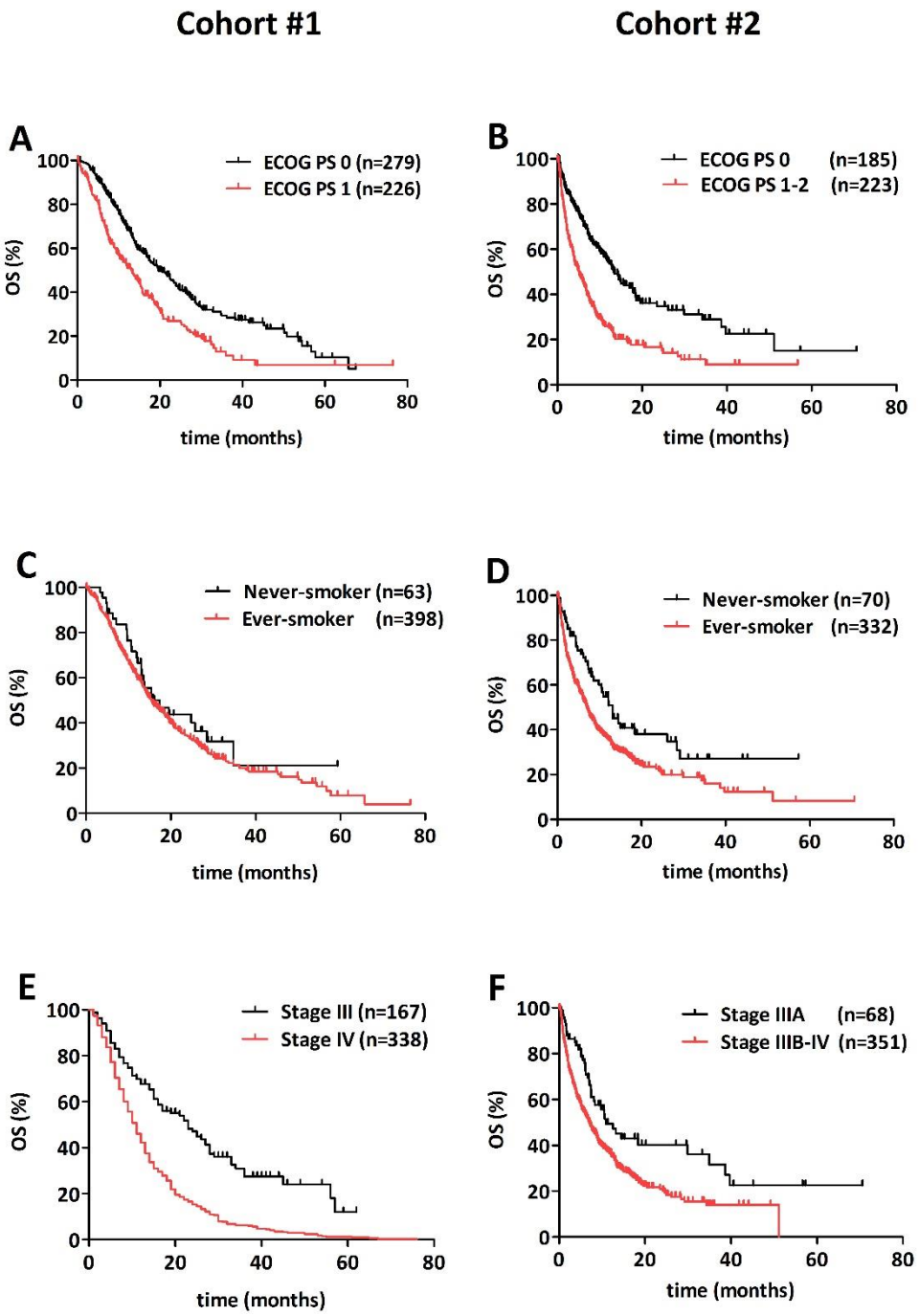


Figure 11. Kaplan-Meier curves for the overall survival (OS) of advanced lung adenocarcinoma patients in cohorts #1 and #2 according to Eastern Cooperative Oncology Group performance status (ECOG PS) (A) ECOG PS 1 (vs. ECOG PS 0; $P<0.001$), (B) ECOG PS 1-2 (vs. ECOG PS 0; $P<0.001$), smoking status (C) we found no difference in OS based on smoking habits in cohort #1 (D) ever-smoker status was a significant prognostic factor in cohort #2 for reduced OS (vs. never-smoker; $P=0.006$), disease stage at diagnosis was prognostic in both cohorts (E) stage III (vs. stage IV; $P<0.001$), and (F) stage IIIB-IV (vs. stage IIIA; $P=0.002$).

Patients with multiple-organ metastases had significantly decreased median overall survival (OS) compared to those with single-organ metastasis (6.8 vs. 11.6 months, respectively; HR, 0.626, 95% CI, 0.498 to 0.788, $P<0.001$, ***Figure 12A***). Next, we compared the prognostic impact of single-organ metastatic sites (***Figure 12B***). Patients with single-organ metastasis to the pleura demonstrated significantly decreased OS when compared to those with lung (median OS, 7.5 v 15.6 months, respectively; HR, 0.460, 95% CI, 0.255 to 0.646; $P<0.001$) or adrenal spread (median OS, 7.5 vs.14.4 months, respectively; HR, 1.896, 95% CI, 1.154 to 3.114; $P=0.011$). Furthermore, patients with brain metastasis showed significantly decreased OS when compared to patients presented with lung metastasis (median OS, 10.3 vs.15.6 months, respectively; HR, 1.5; 95% CI, 1.004 to 2.117; $P=0.04$). We found no statistically significant information in other organ-specific comparison.

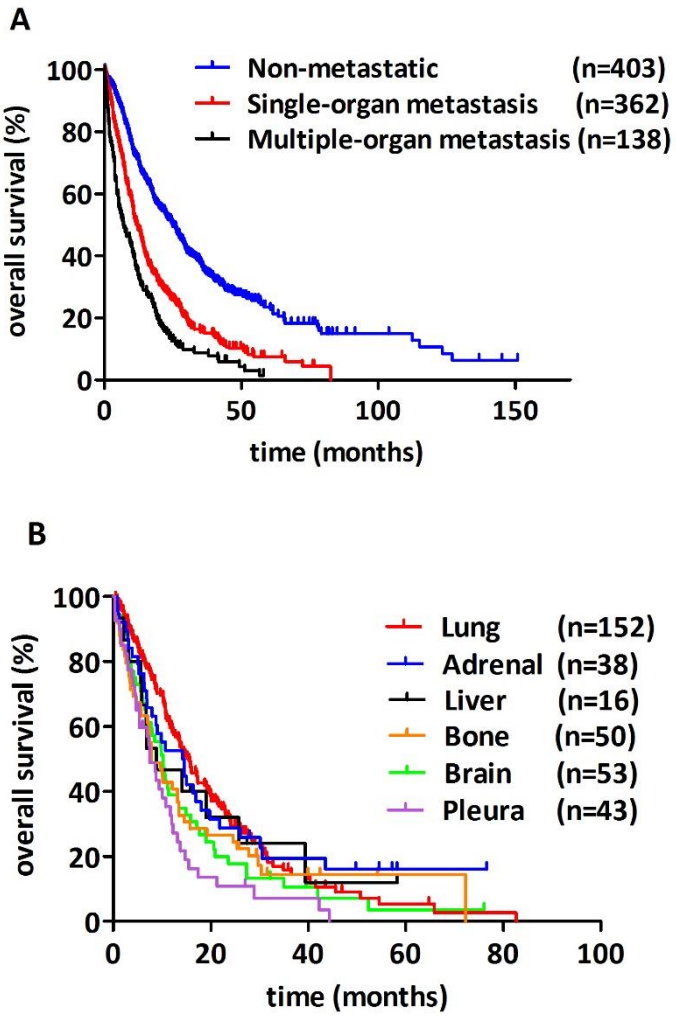


Figure 12. Prognostic impact of metastatic sites. (A) Kaplan-Meier analysis of non-metastatic cases, single-, and multiple-organ metastatic sites, Patients with multiple-organ metastases had significantly decreased median overall survival (OS) compared to those with single-organ metastasis (6.8 vs.11.6 months, respectively; Hazard Ratio (HR), 0.6262, 95% Confidence Interval (CI), 0.498 to 0.788, $P<0.001$). (B) In the comparison of single-organ sites (lung, bone, adrenal, brain, pleura, and liver), patients presented with metastasis to the pleura demonstrated significantly decreased OS when compared to those with lung (median OS, 7.5 vs.15.6 months, respectively; HR, 0.460, 95% CI, 0.255 to 0.646; $P<0.001$) or adrenal spread (median OS, 7.5 vs.14.4 months, respectively; HR, 1.896, 95% CI, 1.154 to 3.114; $P=0.011$). Furthermore, patients with brain metastasis showed significantly decreased OS when compared to patients presented with lung metastasis (median OS, 10.3 vs. 15.6 months, respectively; HR, 1.5, 95% CI, 1.004 to 2.117; $P=0.04$). We found no statistically significant information in any other organ specific comparison.

4.3.2. Prognostic role of EGFR and KRAS mutations in advanced lung adenocarcinoma

Of note, we found no effect of *KRAS* mutational status of tumors on OS in neither cohorts (*P*=0.621, log-rank test; ***Figure 13***). There was no difference between *KRAS* codon 12, codon 13 mutant or *KRAS* WT patients in OS ***Figure 13A***. We also observed no difference in OS according to *KRAS* mutation status in patients presenting with single or with multiple-organ tumor involvement (***Figure 13B***).
However, classic *EGFR* mutation conferred a significant benefit for OS as compared to *EGFR* and *KRAS* WT (HR 0.58; 95% CI, 0.37-0.91; *P*=0.02; ***Figure 13C***) or *KRAS* mutation (HR 0.52; 95% CI, 0.31-0.89; *P*=0.0167; ***Figure 13C***). In contrast, there was no significant difference in the OS of rare *EGFR* mutation positive patients compared to patients with WT *KRAS*/*EGFR* or with mutant *KRAS*.

Next, we investigated the impact of *KRAS* mutation on OS in different organ-specific metastases in lung adenocarcinoma patients (***Figure 14***). We found a clinically relevant and significant increase in OS in patients presenting with *KRAS* WT bone metastasis (vs. *KRAS* mutants, median OS 9.7 vs. 3.7 months; HR, 0.49; 95% CI, 0.31 to 0.79; *P*=0.003; ***Figure 14B***). Importantly, we found no statistically significant information in any other organ-specific comparison.

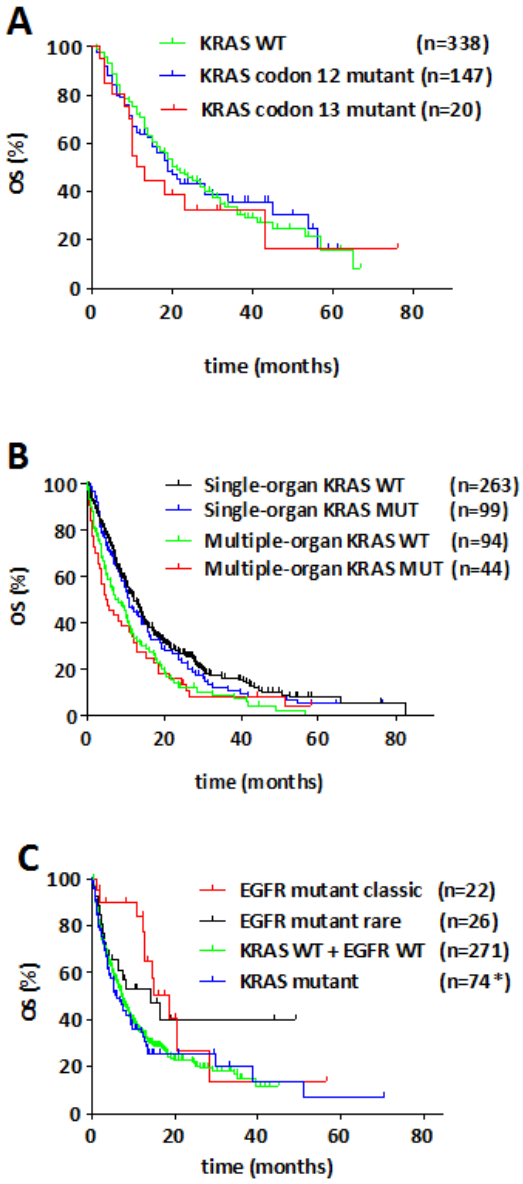


Figure 13. Kaplan-Meier curves for the overall survival (OS) of advanced lung adenocarcinoma patients according to mutation status. (A) *KRAS* mutational status (there was no statistically significant information from these curves in any comparisons (P=0.621, log-rank test, cohort #1). (B) *KRAS* mutational status according to single and multiple-organ spreads (there was no statistically significant information from these curves (log-rank test, combined cohort). (C) Moreover, patients with tumors harboring classic *EGFR* mutations had a significantly longer median OS than those with *EGFR*/*KRAS* double wild-type (WT) (P=0.02) or with *KRAS* mutant (MUT) tumors (P=0.002). Importantly, *EGFR* classic mutation was not associated with benefit in OS if these patients were compared with the rare *EGFR* mutant cohort (P=0.529). * Additionally, in six patient survival data was not available.

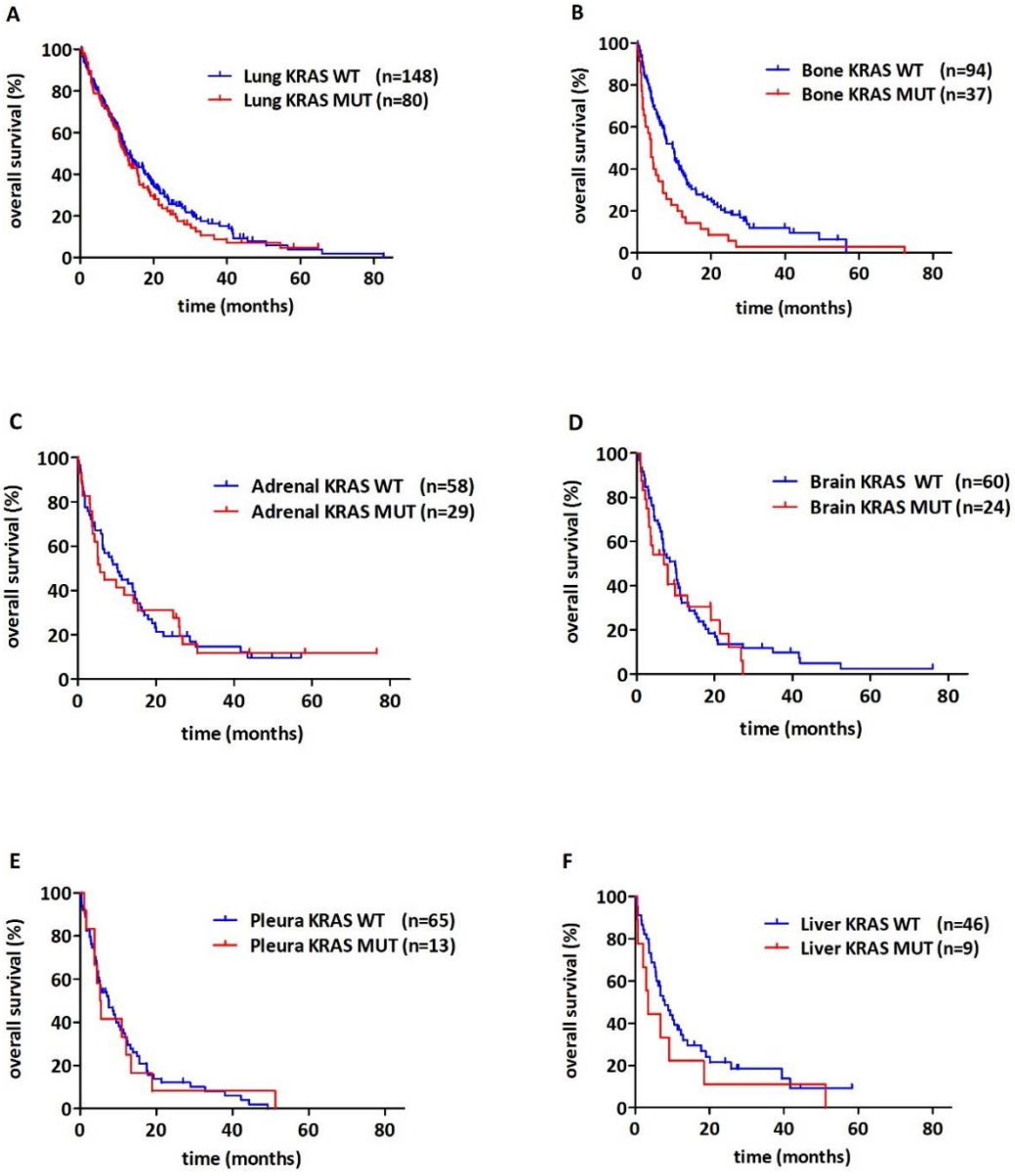


Figure 14. Kaplan-Meier curves for the overall survival (OS) in metastatic lung adenocarcinoma patients in the combined cohort according to *KRAS* mutation status in patients with (A) lung, (B) bone, (C) adrenal, (D) brain, (E) pleura, and (F) liver spread. Both single- and multiple-organ metastases were included in our analyses. We found a clinically relevant and also significant decrease in OS in patients presented with *KRAS* mutant (MUT) bone metastasis (vs. *KRAS* wild-type (WT), median OS 9.7 vs 3.7 months; hazard ratio (HR), 0.49, 95% confidence interval (CI), 0.31 to 0.79; P=0.003; log-rank test). Importantly, we found no statistically significant information in any other organ-specific comparison.

The multivariate Cox regression model in cohort #1 (***Table 8***) identified older age as a significant negative prognostic factor for PFS but not for OS (*P* values were 0.002 and 0.101, respectively). ECOG PS and clinical stage proved to be independent prognosticators for both OS and PFS in a multivariate analysis as well (***Table 8***).

In addition, we found no association between age and OS in the multivariate Cox regression model in cohort #2 (***Table 9A*** and ***B***).

Furthermore, in cohort #2, the Cox model showed that - besides ECOG and stage - classic *EGFR* mutation was an independent survival predictor (HR 0.45; 95% CI, 0.25-0.82; *P*=0.009; ***Table 9A.***). Importantly, rare *EGFR* mutation was not a significant independent predictor of OS (***Table 9B***).

Table 8. Clinicopathological variables and survival of patients with advanced pulmonary adenocarcinoma (n=505) in the Cox proportional hazards model

Prognostic factor	**Overall Survival**		**Progression-free survival**	
	HR (95% CI)	***P value***	**HR (95% CI)**	***P value***
Age (continuous)	0.987 (0.972-1.003)	0.101	0.979 (0.966-0.992)	**0.002**
Gender (male vs. female)	1.213 (0.952-1.546)	0.119	1.055 (0.861-1.294)	0.604
Smoking (never- vs. ever-smokers)	1.208 (0.864-1.688)	0.269	1.127 (0.846-1.502)	0.413
ECOG PS (0 vs. 1)	1.871 (1.463-2.394)	**<0.001**	1.620 (1.310-2.005)	**<0.001**
Stage (III. vs. IV.)	1.487 (1.150-1.924)	**0.002**	1.738 (1.397-2.162)	**<0.001**
KRAS status (WT vs. mutant)	1.020 (0.794-1.310)	0.876	0.962 (0.780-1.186)	0.717

HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status

Table 9. Clinicopathological variables and overall survival of patients with advanced lung adenocarcinoma (n=419) in the Cox proportional hazards model according to (A) classic *EGFR* mutation vs. WT, (B) rare *EGFR* mutation vs. WT.

A

Prognostic factor	**HR**	**95% CI**	***P***
Age (continuous)	0.998	(0.985-1.011)	0.715
Gender (male vs. female)	1.063	(0.820-1.378)	0.643
ECOG PS (0 vs.≥1)	1.320	(1.160-1.503)	**<0.001**
Stage (IIIA vs. IIIB-IV)	1.199	(1.053-1.366)	**0.006**
EGFR status (Classic vs. WT)	0.454	(0.252-0.819)	**0.009**

HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status

B

Prognostic factor	**HR**	**95% CI**	***P***
Age (continuous)	0.999	(0.986-1.013)	0.916
Gender (male vs. female)	1.053	(0.813-1.365)	0.696
ECOG PS (0 vs.≥1)	1.341	(1.184-1.520)	**<0.001**
Stage (IIIA vs. IIIB-IV)	1.157	(1.023-1.310)	**0.021**
EGFR status (Rare vs. WT)	0.730	(0.416-1.279)	0.271

HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; wild-type (WT).

4.4. Therapeutic consequences of subtype-specific oncogenic mutations in advanced lung adenocarcinoma.

4.4.1. Different response to platinum-based chemotherapy with subtype-specific KRAS mutations

According to our inclusion criteria, all patients recieved a platinum-based doublet regimen (unresected stage III patients received chemotherapy in combination with radiotherapy). One hundred and ninety-seven (39%) and 308 (61%) patients were treated with cisplatin and carboplatin, respectively. Platinum was most frequently given together with paclitaxel (58%). Other partners were gemcitabine (31%), pemetrexed (9%), and docetaxel (2%).

There was no difference in ORR or PFS among tumors carrying *KRAS* codon 12, codon 13 mutations or *KRAS* WT (***Supplemental Table 2***).

We evaluated the ORR and PFS of platinum-based chemotherapy treated locally advanced or metastatic lung adenocarcinoma patients with amino acid-specific *KRAS* mutations in codon 12 (***Figure 15*** *and* ***Table 10***).

As mentioned above, we found that G12V *KRAS* mutant patients were significantly more frequent among never-smokers than other codon 12 *KRAS* mutant (G12x) cases (P=0.016, ***Figure 15A***). This subgroup of patients had a non-significantly increased ORR to platinum-based chemotherapy (P=0.077, ***Figure 15B***). Furthermore, there was a non-significant modest increase in PFS. Median PFS in the G12V group was 233 days vs. 175 days in the G12x cohort (P=0.145, ***Figure 15C***). Of note, this difference has diminished in the OS (data not shown).

Table 10. Correlation of outcome variables and *KRAS* codon 12 subtypes in patients with advanced pulmonary adenocarcinoma (n=136)

		G12C (n=61)	**G12V (n=29)**	**G12D (n=27)**	**Rare (n=19)**	***P***
Response	PD+SD	30 (49.2%)	10 (34.5%)	15 (55.6%)	12 (63.2%)	0.219
	CR+PR	31 (50.8%)	19 (65.5%)	12 (44.4%)	7 (36.8%)	
Survival	Median PFS (days)	191 (153-229)	233 (138-328)	150 (91-209)	198 (120-276)	0.135
	Median OS (days)	561 (425-697)	470 (328-561)	325 (165-485)	559 (141-977)	0.801

Data shown in parentheses are column percentages; ECOG PS, Eastern Cooperative Oncology Group performance status; PD, progressive disease; SD, stable disease; CR, complete response; PR, partial response, PFS, progression-free survival; OS, overall survival.

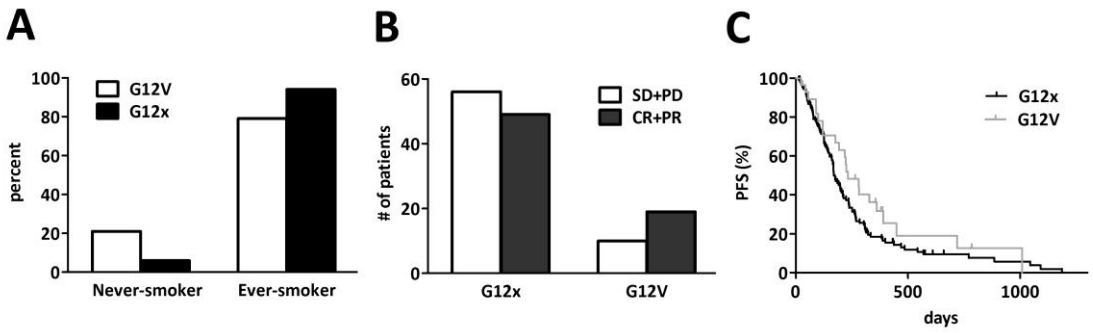


Figure 15. Comparison of (A) smoking history, (B) response rate and (C) PFS of adenocarcinoma patients with G12V versus all the other codon 12 *KRAS* mutations (G12x). A, G12V is significantly more frequent in never-smokers than other codon 12 *KRAS* mutant (G12x) cases (P=0.016, Chi-square test). B, The subgroup of patients with G12V tumors tended to respond better to platinum-based chemotherapy (data presented as number of patients; P=0.077). C, Furthermore, patients with G12V *KRAS* mutant tumors tended to have longer PFS than those with other codon 12 (G12x) mutations (median PFSs were 233 vs. 175 days, respectively, P=0.145). PFS, progression-free survival; PD, progressive disease; SD, stable disease; CR, complete response; PR, partial response.

4.4.2. Different response to TKI therapy in patients with classic versus rare EGFR mutations

In cohort #2, patients received the following TKI therapies: gefitinib or erlotinib monotherapy in 33 or 118 cases, respectively (***Table 11***).

Table 11. Distribution of *EGFR* mutation status in TKI-treated patients.

		TKI therapy		
		Total	**1st line**	**2nd and 3rd line**
Total		151 (100%)	30 (20%)	121 (80%)
Wild-type for KRAS and EGFR		98	0	98 (100%)
Synonymous EGFR mutation		9	2 (22%)	7 (78%)
EGFR mutation	**Total**	44	28 (64%)	16 (36%)
	Classic	24	17* (71%)	7* (29%)
	Rare	20	11** (55%)	9 (45%)

Data shown in parentheses are row percentages.
* In one patient concomitant *KRAS* mutation was identified.
** In two patients, concomitant *KRAS* mutation was identified.
TKI, tyrosine kinase inhibitor.

Next, we evaluated the therapy response, PFS, and OS of TKI-treated advanced lung adenocarcinoma patients with classic and rare *EGFR* mutations (***Figure 16A, B*** and ***C, Table 11***). Irrespective of treatment line, there was a significantly increased ORR among patients with classic *EGFR* mutations compared to those with rare *EGFR* mutations (ORR 71% vs 37%, respectively; P=0.039, ***Figure 16A***). This translated into a statistically not significant but clinically notable longer PFS: the median PFS values were 12.0 and 6.2 months in the classic and rare *EGFR* mutation cohorts, respectively; (P=0.076; ***Figure 16B***). We found no significant difference in the OS in the above mentioned subgroup of patients (P=.212; ***Figure 16C***). Importantly, when classic *EGFR* mutation positive patients were pooled together with patients harboring TKI-sensitizing rare *EGFR* mutations (G719 and L861) [90] and compared to the remaining rare mutation cases, the difference in ORR remained significant (ORR 70% vs 36%, respectively; P=0.044, ***Figure 17A***) and the effect on PFS reached statistical significance (P=0.048; ***Figure 17B***). Importantly, there was a significant difference in the OS in the latter comparison (P=0.01; ***Figure 17C***).

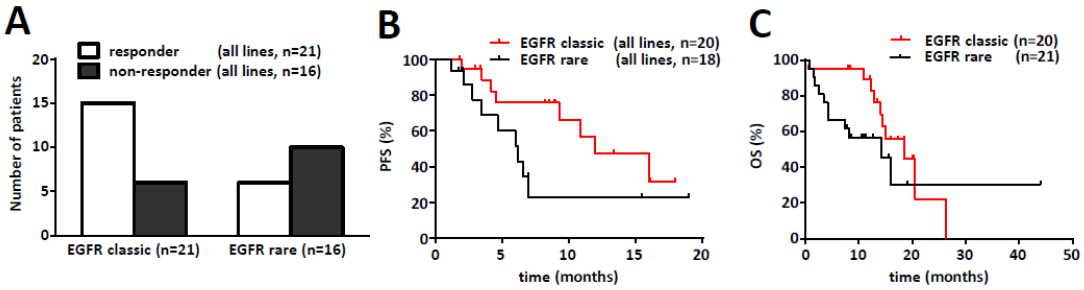


Figure 16. EGFR tyrosine kinase inhibitor (TKI) treatment in advanced lung adenocarcinoma patients with classic versus rare *EGFR* mutations. (A) Irrespective of treatment line, patients with classic *EGFR* mutant tumors responded significantly better to TKI therapy (data presented as number of patients; P=0.039; Chi-square test). (B) Patients with classic *EGFR* mutant tumors tended to have longer progression-free survival (PFS) than those with other rare *EGFR* mutations (P=0.076). (C) There was no overall survival (OS) benefit in patients with *EGFR* classic mutations as compared to those with rare *EGFR* mutations. The discrepancy in the case numbers was due to the lack of availability of retrospective clinical data.

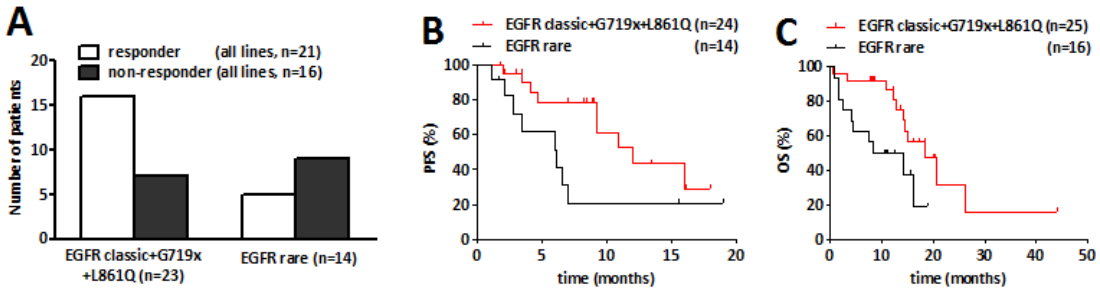


Figure 17. EGFR tyrosine kinase inhibitor (TKI) treatment in advanced lung adenocarcinoma patients with confirmed sensitizing (classic *EGFR* mutations pooled together with patients with sensitizing rare *EGFR* mutations (G719x and L861Q)) versus all other rare *EGFR* mutations. (A) Irrespective of treatment line, patients with sensitizing *EGFR* mutations responded significantly better to TKI therapy (data presented as number of patients; P=0.047). (B) Patients had significantly longer progression-free survival (PFS) and (C) overall survival (OS) than those with other rare *EGFR* mutations (P=0.043, P=0.01, respectively). The discrepancy in the case numbers was due to the lack of availability of retrospective clinical data.

4.5. Oncogenic driver dependent in vitro zoledronic acid sensitivity of lung adenocarcinoma cells

In order to investigate the factors contributing the poor prognosis of *KRAS* mutant bone metastatic patients we performed experiments to test the sensitivity of *KRAS* mutant and *KRAS* WT lung adenocarcinoma cells to ZA, a frequently administered therapeutic regimen in bone metastatic patients.

Therefore, we performed clonogenic assay in lung adenocarcinoma cells following bisphosphonate treatment with ZA (***Figure 18*** and ***Figure 19***)) to investigate long-term effect of 10 days of 1, 2, 8, and 32 μM ZA treatment on clonogenic growth. All cell lines demonstrated sensitivity. Interestingly, resistance was not found in any of the cell lines including *KRAS* mutant cells.

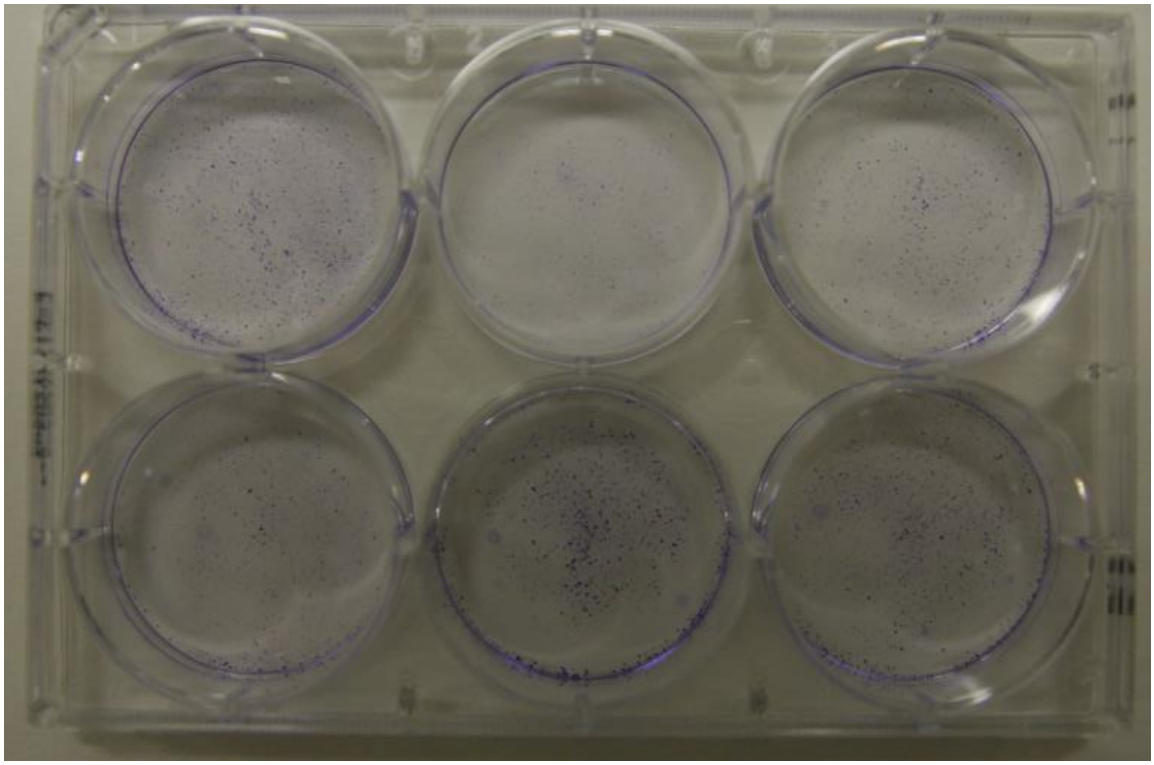


Figure 18. Plate image of clonogenic assay of lung adenocarcinoma cells (CRL5922) following1, 2, 8, and 32 μM zoledronic acid treatment.

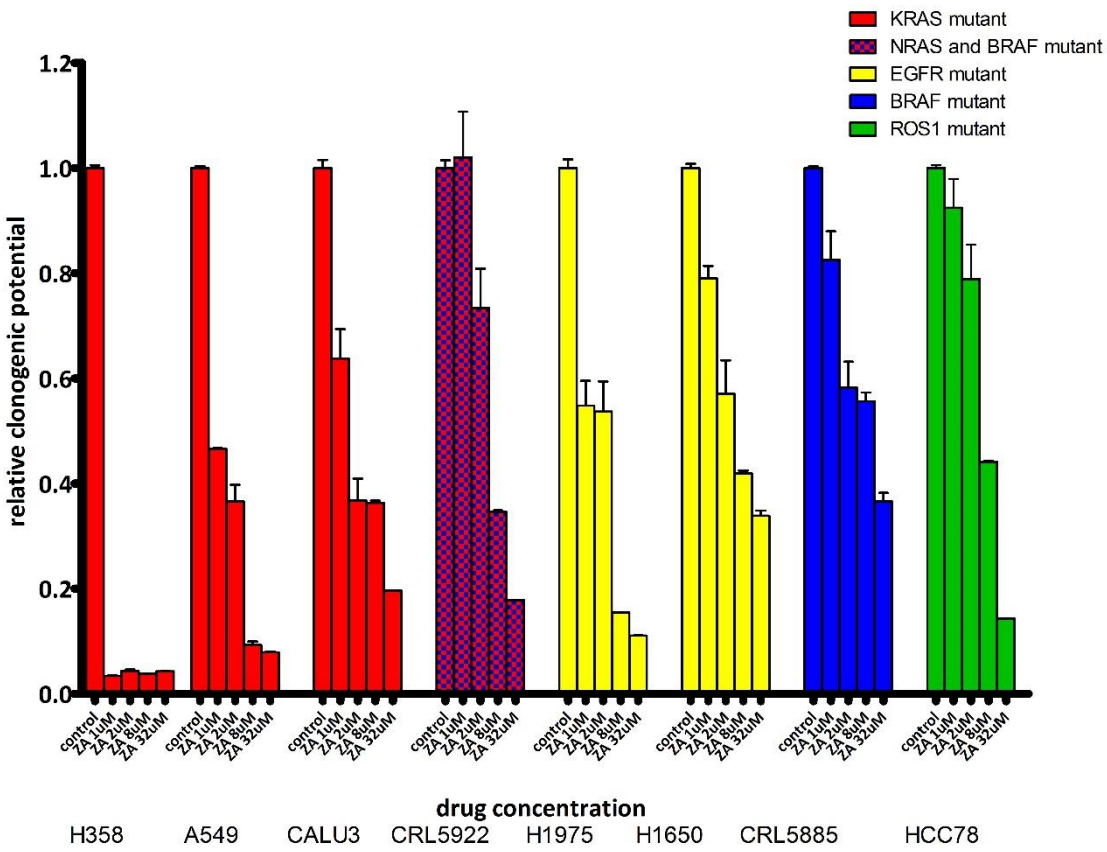


Figure 19. Clonogenic growth of lung adenocarcinoma cells following treatment with zoledronic acid (ZA). Long-term effect of 10 days of 1, 2, 8, and 32 μM ZA treatment on clonogenic growth. While growth was inhibited in all cell lines, the *KRAS* mutant cells did not show reduced sensitivity. *KRAS* mutant (H358, A549 and CaLu-3) and *KRAS* wild- type (CRL5922, H1975, H1650, CRL 5885, and HCC78).

5. DISCUSSION

5.1. Molecular epidemiology of driver mutations in advanced lung adenocarcinoma

In this thesis we discuss the epidemiology and clinical relevance of subtype-specific driver oncogenic mutations, especially in an era where there is an urgent, unmet need to include more lung cancer patients in targeted therapy and other effective treatment regimens. Clinicopathological characteristics of tumors play an important role in therapy decision and help tumor boards to select patients for molecular analysis. A major obstacle to draw a definitive conclusion is the vast heterogeneity of the studies in terms of ethnicity, histological subtype, and tumor stage and treatment modality. Therefore, in the current studies, we analyzed a well-defined Caucasian patient cohort within a three-year-long period. Of note, the very recent INSIGHT Central European study that did not exclude some selection toward patients with higher likelihood of mutation-positive tumors [92]. Furthermore, there are several rare mutations in the *EGFR* gene and subtype –specific *KRAS* mutations with unknown epidemiology.

Importantly, in our study we included all lung adenocarcinoma patients for whom *EGFR* mutational analysis was requested during the period our study covered. Accordingly, it was indeed a consecutive patient cohort.

The *KRAS* mutation rate in cohorts #1, 2, and combined cohort was 33%, 28%, and 29% respectively. This is in line with other large NSCLC studies when case numbers are adjusted for adenocarcinoma [28, 93]. Furthermore, we found a comparable ratio of codon 12 and 13 mutations (93% and 7%, respectively) [93]. We performed Sanger sequencing to evaluate the amino acid substitution-specific subtype of the *KRAS* mutant tumors. Of note, the prevalence of the major subtypes (G12C (38.6% and 42%), G12V (18.4% and 20%), G12D (17.1% and 15%) and G12A (5.1% and 7%)) were similar between our study and in the COSMIC database [91], respectively (***Table 12***).

Table 12. The most frequent amino acid substitution-specific mutations of *KRAS* in lung adenocarcinoma.

Nucleotide change	Amino acid change		Abbreviation	COSMIC	Cohort #1*
GGT>TGT	Glycin	Cysteine	G**12**C	42%	39%
GGT>GTT	Glycin	Valine	G**12**V	20%	18%
GGT>GAT	Glycin	Aspartic acid	G**12**D	15%	17%
GGT>GCT	Glycin	Alanine	G**12**A	7%	5%

*In 31 cases rare KRAS codon 12 and 13 subtype mutations were identified.
COSMIC: Catalogue of somatic mutations in cancer.

Regarding *EGFR* mutations, in our patient population we separated the synonymous (or also called silent) *EGFR* mutations because they do not result in amino acid change. Accordingly, we used the term rare mutations only for non-classic mutations where an amino acid change occurs. Of note, synonymous (silent) mutations were not reported among rare or uncommon mutations in several previous papers [59, 94]. In order to underline this distinction, the rows of synonymous mutations are highlighted in ***Supplemental Table 1***.

In cohort #2, five percent of patients carried classic *EGFR* mutation. In a recent Caucasian study, the incidence of confirmed activating *EGFR* mutation in lung adenocarcinoma patients was reported to be 6% [6, 66]. The incidence of rare non-synonymous *EGFR* mutations in our cohort was 6% and therefore is higher than in other Caucasian studies (1.9%-2.7%) [66, 94] or in a mixed US study population (4%) [95]. However in line with East-Asian studies, the incidence of rare *EGFR* mutations ranged from 7% to 8% [90, 96, 97]. The higher proportion of rare mutations in our Caucasian cohort is likely because Sanger sequencing of exon 20 was also performed (in 76% of the patients) and that 40% of all *KRAS* mutant cases underwent *EGFR* analysis as well. However, these arguments do not fully explain the high rate of rare *EGFR* mutations. Indeed, it has been reported in both Asian and Caucasian studies, that about 90% of all lung NSCLC-associated *EGFR* mutations are classic ones whereas the proportion of rare *EGFR* mutations usually does not exceed 10-15% [98, 99]. We need to be aware of the fact that the sensitivity and

specificity of the different molecular tests can vary. In addition, there are differences in epidemiology of rare *EGFR* mutations in different patient populations. Based on histology, ethnicity, and environmental factors, the incidence of certain molecular alterations can highly vary. A recent retrospective study from North Africa recently published the rate of rare *EGFR* mutations at 10% of all EGFR mutations [100].

Since there is limited data available from Africa, this was a unique opportunity to highlight differences in epidemiology of rare *EGFR* mutations in contrast to a patient population reported from North Africa.

The complete coverage of exons 18 to 21 and the *EGFR* analysis in *KRAS* mutant patients can very well be one reason for the increased rate of rare *EGFR* mutations. Additionally, smoking status can also have an influence on the high frequency of rare *EGFR* mutations. This impact may depend on patient population. In our patient cohort, the frequency of smokers was very high, thus leading towards enrichment for rare *EGFR* mutations. Interestingly, the rare *EGFR* mutations in Asian populations do not appear to be linked to smoking, in contrast to Caucasian cohorts. Importantly, the epidemiology of rare EGFR mutations in Morocco resembles more an Asian population than Caucasian study cohorts [101].

It cannot be emphasized enough that the absence of identical molecular methods is even more delicate to match side-by-side the different studies. A number of commercial mutation analysis methods demonstrate increased sensitivity but only for a preselected set of molecular alterations that might enrich for classic *EGFR* mutations [34]. In contrast, Sanger sequencing have a low sensitivity towards classic *EGFR* mutations when compared to targeted molecular methods like HRM or Therascreen. As mentioned in the Methods section of the thesis, in our study, the most frequently used molecular method was Sanger sequencing. The sensitivity is approximately 20% (it is able to detect mutations in specimens with at least 20% cancer cell content).

In our study in seven cases, the Therascreen EGFR29 Mutation Kit was used. This assay is able to detect 29 mutations including classic and certain previously identified rare mutations in exons 18, 19, 20 and 21 of the *EGFR* gene [34]. In our cohort, the Therascreen assay identified only WT patients, therefore we are not able to compare Therascreen and other *EGFR* mutation testing methods.

Furthermore, a possible reason for the discrepancy between our analysis and other studies can be that several studies include only a relatively low number of patients (n=100-300) and/or the use of targeted molecular methods or different patients population. Furthermore, the lack of outcome data in some studies may make the translational research and the validation process impossible. In addition to the above-mentioned facts, similarly to other studies, the epidemiology of rare mutations was rather a descriptive part of our study. Like most of the translational studies, we could only hypothesize the biology and the background of our findings. More importantly, outcome data published along with molecular findings are of crucial interest and greatly assist molecular pathologists in the validation process of data generated by different molecular methods. Of note, the same problem we are facing currently, is the clinical utility of PD-1 and Programmed death-ligand 1 (PD-L-1) antibodies. In addition, recent data from the World Conference on Lung Cancer (WCLC) 2015 highlighted in lung cancer (and malignant melanoma) the number of mutations present in the tumor associated with immunotherapy efficacy. Therefore, whenever possible, it is very important to report outcome data along with molecular epidemiology.

In addition, in our study, we found simultaneous (concomitant) or in other words complex (at least two different *EGFR* mutations in one sample) gene mutations. In seven patients, concomitant *KRAS* and classic or rare *EGFR* mutations were identified. These patients are 1.2% (7/584) in the group of patients with both *KRAS* and *EGFR* mutation analyses. This ratio is in line with already published studies [30, 59]. Of note, 2% of our patients carried complex mutation pattern, meanwhile an East-Asian study published 7.3% [97]. To our knowledge, no Caucasian population-based study has reported the comprehensive frequency of complex *EGFR* mutations yet. We were not able to detect the resistance-associated mutation (T790M) in our patient cohort. This is in line with its very low incidence (0-0.9%) in previous analyses of tumors before TKI therapy administration. These studies used molecular methods that lacked increased sensitivity towards mutant alleles [17, 102]. In contrast, studies enriching for mutant alleles using a peptide-nucleic acid to inhibit the amplification of WT allele found much higher incidence of pretreatment T790M resistance mutations (35-65%) [65, 103].

According to our best knowledge, our study is among the first to compare the age between rare and classic *EGFR* mutants, *EGFR* and *KRAS* WT, and *KRAS* mutant patients in a Caucasian cohort. In cohort #2, patients with classic *EGFR* mutations tended to be older (mean age: 67±9.6 years) than those with rare *EGFR* mutations (mean age: 64.2±9.2 years) and were significantly older than patients harboring *KRAS* mutations (mean age: 60±10.4 years). In line with the latter findings, in cohort #1, one-way ANOVA test with Tukey Multiple Comparison indicated a significant difference between the average ages of *KRAS* WT and codon 12 mutant patients (60.7 versus 58.8 years, respectively, P=0.032). Accordingly, the above mentioned recent German study also found an almost significant trend between patients with *KRAS* (mean age: 65.3±9.8 years) and *EGFR* mutations (mean age: 70.3±11.4 years) [66].

Importantly, in contrast to studies of East-Asian origin, we demonstrated in our Caucasian population that patients harboring *KRAS* mutations are younger than those with classic *EGFR* mutations. This finding is in line with a study on an East-Hungarian patient population from the University of Debrecen (Ostoros et al., unpublished data).

We found no correlation of age, and *KRAS* exon 2, codon 12 mutation subtypes in patients with advanced pulmonary adenocarcinoma.

We found no correlation of gender and any mutations detected. Furthermore, significant associations between gender and rare *EGFR* mutational status were not found in our cohort #2 in line with a very recent – and to date the only similar – Caucasian study [104].

In NSCLC, *KRAS* exon 2, codon 12 is recognized as a preferential site for cigarette smoke-induced mutagenesis, and thus mutations in this codon are more common in tumors of ever-smokers [105, 106]. Codon 12 *KRAS* mutation in our cohort #1 and 2 was also significantly associated with cigarette smoking. Interestingly, however, we found that never-smokers were significantly more likely to have a G12V transversion mutation than other subtypes of codon 12 mutation. This observation is not in line with previous studies [13, 105, 107-109] where G12D appeared to be the most frequent mutation among never-smokers compared with other codon 12 mutation subtypes.

Although the reasons for this discrepancy between the above studies and our cohort are unclear, the difference might be explained by ethnic factors since we analyzed patients only of Caucasian background whereas the above studies included mixed US cohorts [13, 105, 109] or patients with East-Asian [107, 108] origin. Nevertheless, our finding raises the possibility that not all subtypes of codon 12 *KRAS* mutations are associated with smoking in Caucasian adenocarcinoma patients.

In our cohort #2, rare *EGFR* mutations appeared to be associated with smoking status when compared to classic EGFR mutations. Our finding is similar to another report that showed that among smoker patients the frequency towards rare EGFR mutations was higher, although not significantly, when compared to never-smokers (20.8 vs. 8%, respectively) [94]. A mixed ethnical population based study demonstrated that among *EGFR* exon 20 insertion mutant patients the frequency of smokers was higher than in patients harboring classic *EGFR* mutations [95]. In contrast, studies from East-Asia showed that rare *EGFR* mutations pooled with complex rare EGFR mutations are linked to never-smokers, [97] and that uncommon (rare) mutations are higher among never-smokers [90].

5.2. Molecular diagnostics of oncogenic drivers

KRAS is a downstream member of the *EGFR* signaling, and therefore *KRAS* mutation is an established negative predictor for TKI therapy. However, routine *KRAS* mutation testing is currently not recommended and the demonstration of activating *EGFR* mutation is needed for TKI therapy indication [27].

Nevertheless, as previously mentioned in the introduction and in the methods section, in Hungary *KRAS* testing is performed at first to exclude *KRAS* mutant cases from *EGFR* analysis as part of a diagnostic algorithm elaborated to reduce costs and to optimize testing and therapeutic efficiency. This screening strategy allows analyzing large numbers of cases for *KRAS* mutations. Furthermore, this approach made our study unique and enabled us to study a more homogenous and well-defined molecular subsets of tumors.

Thus, we were able to compare *EGFR* mutant, *KRAS* mutant, and *EGFR*/*KRAS* double WT patient cohorts.

There is no comprehensive data and guidelines lack comprehensive information on the molecular diagnostics of lung adenocarcinoma. Importantly, epidemiological studies with sensitive methods are needed to establish the incidence of targetable molecular alterations. In the French study (ERMETIC), it was determined that the quality and type of the sample has a great influence on the outcome of a molecular analysis [104]. In poor-quality samples, DNA concentration cannot be determined accurately. Any tumor sample from which DNA can be recovered is suitable for analysis, and should contain a sufficient amount of tumor cells. The ratio of tumor tissue in samples ranges from 5 to 100%. Less than 20% is usually not enough (Sanger sequencing) for appropriate sensitivity. Similarly, mutant DNA content should not be lower than 20% for detecting mutation by direct sequencing. The tumor cell content of the samples can be enriched by macrodissection or laser microdissection, which can increase efficacy but can be expensive and time consuming. HRM, capable of detecting mutant DNA at a percentage as low as 2.5% to 10% and is not too expensive, can be an alternative; however, the result must be confirmed by direct sequencing.

While thin needle biopsies - frequently used in thoracic oncology - may have a high ratio of tumor cells, pleural fluids usually contain low quantities of tumor cells. In the case of low tumor cell ratio, techniques of higher sensitivity, such as mutant-enriched PCR or amplification refractory mutation system (ARMS), should be used.

In the majority of the cases, *EGFR* mutations were successfully identified by direct sequencing on samples obtained from the lung by transthoracic punction (TTP), endobronchial ultrasound guided biopsy (EBUS) or CT guided biopsy [110].

Worldwide FFPE tumor tissues are available and almost exclusively used in oncology for diagnostic purposes. Furthermore, the diagnostics are commonly made on tumor biopsy samples. In the last decade, several scientific meetings and guidelines did not conclude which *EGFR* mutations should be tested or which methods should be used. Accordingly, to date, it is not clear which molecular technique is the most appropriate with regards to sensitivity, specificity, and reproducibility. Addiotional important aspects can be the requirement for short turnaround time or low input DNA. Other pathological factors such

as presence of lymphocytes, necrosis or mucin content in tumors can also influence the quality and interpretation of results [34].

Also, we must be aware that the diagnosis of advanced NSCLC is more commonly made by biopsy rather than surgically resected tumor samples.. Indeed, throughout the world the majority of molecular testing is performed on FFPE surgical tumor specimens or biopsies, or even on cytological preparations. However, fresh frozen tumor sample is considered one of the most appropriate for DNA isolation.

The current routine practice can lead to the detection of artifactual mutations, specifically to the emergence of formalin-fixation-related PCR artifacts. In our study, rare mutations were all identified from samples of DNA extracted from FFPE tissue. Artifacts can occur when sequencing multiple PCR amplification products of very small amounts of DNA. Large-scale DNA fragmentation and base damages like cytosine deamination can be caused by the chemical reactions during formalin fixation. Thereby the so-called "A-rule" can happen when the taq DNA polymerase insert an adenosine as a substitute of a guanosine resulting in C->T and G->A transitions. Moreover, degraded PCR products allow the taq DNA polymerase to perform a "jump" from a damaged template to another to continue the extension [111].

In our cohort, the majority of the rare *EGFR* mutations identified have already been published in the COSMIC database. Additionally, twenty previously not published rare *EGFR* mutations were identified, (among them three microdeletions and five point mutations were found) which were not C->T or G->A transitions that often appear as formalin induced artifacts. Of note, five patients with novel rare *EGFR* mutations responded to therapy and demonstrated a survival benefit that would not be expected in the case of artifact mutations or in *EGFR* WT patients. In two cases (harboring the P848S mutation and L852R with PR) cytology sample was available, but for the three other patients histological sample was available, consequently the likelihood is high that sufficient amount of tumor DNA was used in the molecular analysis. Furthermore, we can exclude the presence of artifacts in specific genetic alterations including deletions, insertions and in a mutation that resulted in stop codon.

Standardization of fixation and other tissue processing procedures can minimize and consider artifacts by establishing what procedures introduce which kinds of artifacts. By expecting the types of artifacts in each tissue type and using a specific technique might enable us to accurately interpret molecular data [34]. Moreover, several other strategies can help to prevent artifactual mutations. Routine application of microdissection to enrich tumor-cell DNA or use of fresh-frozen tissue can also improve the testing efficiency. Also, if small amounts of DNA extract from FFPE a inevitable, after PCR amplification, addition of uracil-N-glycosylase to the DNA and the examination of multiple amplifications are crucial.

Nevertheless, we hope that because an increasing number of *EGFR* mutation analyses are being performed on non-formalin-fixed specimens the spectra of validated somatic *EGFR* mutations will eventually be established. Novel diagnostic methods like liquid biopsy (circulating tumor DNA) may also help in a more accurate diagnosis in the future [112].

5.3. Prognostic factors in advanced lung adenocarcinoma

With regard to factors associated with OS in lung adenocarcinoma, we confirmed in cohort #2 the prognostic significance of gender, ECOG PS, disease stage, in line with the findings of others [113]. Similarly, in cohort #1, disease stage and ECOG PS was found to be associated with longer OS. In contrast to cohort #2, in cohort #1, we found no difference in OS according to smoking status and gender. In cohort #2, we found significantly increased OS among never-smokers as compared to ever-smoker patients. This discrepancy may be due to the fact that in cohort #1 the inclusion criteria was based on *KRAS* mutation analysis and treatment with platinum-based chemotherapy, meanwhile in cohort #2, *EGFR* (and/or *KRAS*) molecular test, and therefore higher percentage of patients (n=150, ***Supplemental Table 4***) received EGFR-TKI therapy, which may influence overall survival.

Furthermore, in cohort #2, in line with others [113], the presence of classic *EGFR* mutations had a statistically significant effect on OS.

We observed no difference in response rate or survival benefit between *KRAS* mutant or *KRAS* WT patients treated with platinum-based chemotherapy. Similarly to the majority of previous publications [27], we were not able to confirm the prognostic effect of *KRAS* mutations. Accordingly, our findings are in line with the TRIBUTE trial that evaluated a similar patient cohort and all patients that received platinum-based chemotherapy [93]. Similarly, neither a retrospective study of 161 NSCLC cases [114], nor a prospective study of 83 NSCLC patients with advanced adenocarcinoma [115] showed significant difference based on *KRAS* mutation status in OS when treated with platinum-based doublet chemotherapy regimens. Nevertheless, it is also important to mention that there was no significant OS benefit in the relatively smaller subset of patients (n=17) with codon 13 mutations in the study of Villaruz et al. on another largely early clinical stage cohort of adenocarcinoma patients [116]. Also, a recent study including 677 *KRAS* mutant patients did not find significant difference in survival between patients with *KRAS* codon 13 versus codon 12 mutations (1.0 versus 1.1 years, respectively) [109]. Of note, an independent validation of tumors from 682 patients with stage IV *KRAS* mutant lung cancers was performed and demonstrated the same outcome. However, a meta-analysis of four randomized trials (including the JBR.10 trial [117] which is a study conducted in an early stage NSCLC population) found that *KRAS* codon 13 mutation (24 patients were evaluated at codon 13) may be a negative predictor of survival after adjuvant chemotherapy [13].

In our study, we found no evidence of such an interaction. Of note, an investigation into differences in the effect of chemotherapy on PFS based on *KRAS* codon and/or substitution types was not performed in the already published studies of advanced-stage NSCLC [108, 109].

In another study of (mostly) early clinical stage NSCLC patients, the authors could not demonstrate an association between amino acid subtype-specific *KRAS* mutations and OS [116]. However, another Caucasian study on resected lung adenocarcinoma patients with *KRAS* G12V exhibited worse OS and higher recurrence incidence [118].

A study of pooled resected NSCLC patients also suggested that different *KRAS* codon 12 amino acid-specific KRAS mutations are neither prognostic nor predictive for adjuvant chemotherapy [119]. Interestingly, this latter study found a negative prognostic effect for chemotherapy in *KRAS* codon 13 mutant cases.

5.4. Clinical relevance of subtype-specific oncogenic mutations in advanced lung adenocarcinoma

Preclinical data suggested that subtype-specific *KRAS* codon 12 mutations in lung adenocarcinoma have distinct biological consequences and may influence the sensitivity of tumor cells to different treatment modalities [12]

Although it has been demonstrated in colorectal carcinoma that *KRAS* G12V transversion leads to poor therapy response and survival [120], the clinical relevance of amino acid-specific KRAS mutations at codon 12 remains to be established in advanced-stage lung adenocarcinoma. In the two recent and so far largest studies of early clinical stage adenocarcinoma, neither the effect of chemotherapy on PFS nor the OS of patients differed among the subpopulations with various codon 12 subtypes [13, 116]. Additionally, other studies on advanced-stage NSCLC failed to demonstrate significant association between *KRAS* codon 12 subtypes and OS [108, 109]. However, the predictive value for chemotherapy benefit among the subpopulations with different codon 12 subtypes was not investigated in the latter studies.

In our cohort #1, patients with G12V amino acid-specific subtype KRAS mutant lung adenocarcinoma patients tended to have better ORR to platinum-based chemotherapy and were more likely to have a non-significantly longer median PFS than those with other codon 12 amino acid-specific KRAS mutants. Our data is in line with growth inhibition assay reported by another group that found powerful differences in response to cisplatin among KRAS overexpressing clones of human lung adenocarcinoma cells (NCI-H1299) with different amino acid substitutions [12]. All in all, the observation of Garassino et al. that G12V mutant cells demonstrated increased response to cisplatin chemotherapy (whereas the most common G12C transversion showed the least response).

Based on our results, we can hypothesize that lung adenocarcinoma patients carrying different subtype-specific *KRAS* mutations might have distinct response patterns to platinum-based chemotherapy. Moreover, that subtype-specific mutation analysis may help to identify the most effective treatment regimen for each individual patient.

To our knowledge, our cohort #2 is the first Caucasian population-based advanced-stage disease cohort with ORRs rates for first-generation EGFR-TKI-treated rare *EGFR* mutation positive lung adenocarcinoma patients. In our study, ORRs were 71% and 37% among patients with classic and rare *EGFR* mutations, respectively. This finding is in line with studies from East-Asia where ORRs were found to be 74-75% and 28-48%, respectively [90, 97]. Interestingly, in the LUX-Lung 2 phase II trial of the second-generation covalent EGFR-TKI afatinib, similar results were found as well (ORR of classic and rare *EGFR* mutant cohorts were 66% and 39%, respectively) [96]. Of note, the 12-months median PFS among classic *EGFR* mutant patients in our cohort #2 is rather similar to previously published data (9.4-11.9 months) from other studies [97, 121, 122]. Patients in our cohort with rare *EGFR* mutations demonstrated a shorter median PFS of 6.2 months. This is comparable to the 5-months median PFS of rare *EGFR* mutant patients in a recent East-Asian study performed by Wu et al. [90]. Importantly, when patients with classic *EGFR* mutations were pooled with patients with rare sensitizing *EGFR* mutations (G719 and L861) and then this cohort was compared to the remaining rare mutation harboring population, the effect on PFS reached significance. Interestingly, a similar robust difference was found in the recently published LUX-Lung 2 clinical trial [96]. Of note, the ORR and PFS in our patient cohort with rare *EGFR* mutations (PFS: 7.4 months; ORR: 31%) is comparable to that of the cisplatin-pemetrexed combination arm in the LUX-Lung 2 clinical trial (PFS: 6.9 months; ORR: 23%) which is now considered one of the most effective chemotherapy regimens in lung adenocarcinoma [64].

Classic *EGFR* mutations were associated with a significantly better median OS when compared to rare *EGFR* mutations (20.5 v 7.4 months) in the current study. This finding is in line with the results of other studies (19.3-20 months) on classic *EGFR* mutation positive cohorts, but differs in the case of rare mutations (9-17 months), possibly due to the different types and proportion of rare *EGFR* mutations [90, 94, 97]. Moreover, not all rare mutations are resistant. It has been reported that some non-classic mutations are highly sensitizing to EGFR-TKI [90].

5.5. Metastatic site-specific variation of KRAS status in lung adenocarcinoma

Despite extensive research, the prognostic and predictive power and thus the clinical utility of *KRAS* oncogenic mutations in lung adenocarcinoma has not yet been defined for over a decade [28, 45]. Surprisingly, there is very limited comprehensive data available regarding the influence of *KRAS* mutation on the organ specificity of lung adenocarcinoma metastases [123].

Patients with multiple-organ metastases showed a modest increase in the incidence of *KRAS* mutations. While there is no published data for lung adenocarcinoma, significantly increased frequency of *KRAS* mutation in multiple organ metastases was found in a colorectal cancer study [124]. Concerning the metastatic sites, in line with previous findings of others [123], in our study patients with brain, bone, or adrenal gland metastases demonstrated similar *KRAS* mutation frequencies. Our study found 28% *KRAS* mutation in the bone metastatic cohort which is similar to previous findings reported by other groups [125, 126]. However, we found pleural dissemination and liver metastasis associated with decreased and intrapulmonary with increased *KRAS* mutation incidence. Interestingly, similar to our study, in colorectal cancer RAS mutation was associated with increased lung [127, 128], and decreased metastatic spread to liver [124, 129].

In line with other studies, we found a significant decrease of median OS in patients with multiple-organ metastases [130]. Our finding further supports the proposal that the M stage should take into account the number of metastases [131].

Comparing single-organ metastatic cases, we found that patients that presented with metastasis to the pleura and brain showed significantly decreased OS when compared to patients exhibited lung metastasis. Earlier studies also showed that patients with metastasis to the brain have an increased negative impact on survival [132, 133].

In our study we directly compared the prognostic role of *KRAS* mutations in the distinct metastatic sites in lung adenocarcinoma. Importantly, we found a clinically relevant and significant increase in OS in patients with *KRAS* WT bone metastasis. The differences between the clinicopathological characteristics of *KRAS* WT and *KRAS* mutant bone-metastatic patients cannot explain the observed decrease in OS. Of note, we found higher

frequency of multiple-organ metastases in *KRAS* WT patients presenting with bone metastases (84% vs. *KRAS* mutant, 46%, $P<0.001$).

With regards to the role of smoking in pulmonary metastasis, we found no association between metastatic pulmonary nodules and smoking. Smoking was found not to be a significant risk factor in developing lung metastases in colorectal cancer [134-136]. In contrast, in esophageal and breast cancer, smoking appears to be associated with pulmonary spread [137, 138]. The number of never-smokers increased in patients with pleural spread (27%) and decreased among liver metastatic patients (8%).

Despite enormous attempts, the prognostic value and the clinical utility of the most frequently occurring oncogene have not been recognized for over a decade. Consequently, guidelines lack information on the clinical benefit of *KRAS* mutation testing in NSCLC. Therefore, and more importantly, our study addresses an important issue and highlights the possible prognostic importance and potential clinical relevance of *KRAS* mutation. In addition, our study is the first that showed metastatic site-specific variation of the prognostic value of *KRAS* status in lung adenocarcinoma. We suggest the *KRAS* mutation may have important implications for diagnostic strategies and treatment decisions. Based on our results, *KRAS* mutation has a strong prognostic value in bone metastatic patients associated with decreased OS. Nevertheless, further studies are needed to evaluate whether *KRAS* mutation can be used to risk stratify patients with bone metastasis or even might predict response to various treatment options for bone metastatic patients.

Since ZA treatment is frequently used in bone metastatic patients and administered frequently in the current study population, we evaluated the in vitro inhibitory effect of ZA on lung cancer cells. We performed clonogenic growth in lung adenocarcinoma cells following bisphosphonate treatment with ZA. All cell lines demonstrated sensitivity. Interestingly, no significant resistance was found in any of the *KRAS* mutant cell lines.

Prenylation inhibition may not depend on the driver oncogenic mutations present in tumor. Importantly, prenylation inhibition may be able to inhibit both KRAS mutant and KRAS WT lung cancer cells. In contrast to our finding, Garay et al. showed benefit of prenylation inhibition may strongly depend on the driver oncogenic mutations present in melanoma cells [139].

The worse outcome of bone metastatic KRAS mutant patients in our combined cohort might not be due to the decreased sensitivity of tumor cells to ZA. Nevertheless, further studies are needed to clarify the clinical relevance of KRAS status in bone metastatic patients.

5.6. Limitations of our retrospective studies

Like all retrospective analyses, our studies have several limitations. Our cohorts are among the largest ones in the corresponding settings. Despite the initial size of the cohort, as expected the final number of patients with subtype-specific mutations was relatively small. Our study provided the possibility to draw some conclusions that clearly need to be validated in subsequent studies. Furthermore, due to the studies' retrospective nature, our major results need to be confirmed in a prospective setting.

Also, we need to be aware of the correct definition of prognostic power. Since our retrospective study cannot distinguish between the treatment-associated increase in survival and the purely prognostic effects. Our study did not include a control group without platinum-based chemotherapy and thus a possible prognostic role cannot be distinguished from a predictive value of specific *KRAS* mutation subtypes on chemotherapy response. Furhermore, it remains unclear whether the classic *EGFR* mutation itself confers a more benign behavior or the increased response rate and median PFS of the classical mutant cohort translates to better prognosis.

Another important potential confounding factor is smoking status, as several studies have demonstrated that never-smokers have improved OS [69, 70]. In our cohort #2, we found a significant overall survival advantage for never-smokers and at the same time, the classic *EGFR* mutant cases were significantly more frequent among never-smokers than rare *EGFR* mutant ones. Thus, it is likely that the increased survival is owing to the overall better performance and the lack of smoking related co-morbidities [69-72].

With regards to the composition of “WT” groups in our studies it is important to emphasize that these patients were not analyzed for additional oncogenic driver mutations. In the combined cohort analysis, we excluded *EGFR* mutants in order to avoid the potential positive prognostic role of EGFR mutation. In addition, we were not able to exclude the presence of asymptomatic disease or micro metastases in the combined cohort since we used the clinical TNM stage. Of note, at the relatively less frequent metastatic site with the lowest *KRAS* mutation incidence, namely in the liver metastasis subgroup, we do not have sufficient statistical power to determine the impact of *KRAS* mutation on overall survival.

Thus, altogether, to address the above limitations, additional large lung adenocarcinoma cohorts should be analyzed. The integration of NGS into routine molecular diagnostics can generate extensive data of subtype-specific mutations in subsequent studies. This will provide the opportunity to study even larger cohorts of patients.

6. CONCLUSIONS

Considering the results of this thesis the following main conclusions can be drawn in order to answer the questions formulated as the aims of the thesis.

1. In lung adenocarcinoma, the G12V subtype of *KRAS* mutations is associated with different clinicopathological characteristics and patients carrying G12V mutations may show increased response to platinum-based doublet regimens.

2. In our study, in lung adenocarcinoma the majority of rare *EGFR* mutations was associated with smoking, shorter overall survival, and decreased EGFR-TKI response when compared with classic *EGFR* mutations. Studies characterizing the EGFR-TKI sensitizing effect of individual rare mutations are indispensable to prevent the exclusion of patients with sensitizing rare *EGFR* mutations who may benefit from anti-EGFR therapy.

3. Our study is the first that showed metastatic site-specific variation of the prognostic value of *KRAS* status in lung adenocarcinoma. We suggest the *KRAS* mutation may have important implications for diagnostic strategies and treatment decisions.

4. Based on our results, we suggest that *KRAS* mutation has a strong prognostic value in bone metastatic lung adenocarcinoma patients associated with decreased OS. Nevertheless, further studies are needed to evaluate whether *KRAS* mutation can be used to risk stratify patients with bone metastasis or even might predict response to various treatment options for bone metastatic patients.

5. The effect of zoledronic acid treatment on the clonogenic potential of NSCLC cell was not dependent on *KRAS* mutant status and thus prenylation inhibition may not depend on the driver oncogenic mutations present in the tumor. Importantly, prenylation inhibition may be able to inhibit both *KRAS* mutant and *KRAS* wild-type lung cancer cells. The worse outcome of bone metastatic *KRAS* mutant patients in our combined cohort might not be due to the decreased sensitivity of tumor cells to zoledronic acid.

7. SUMMARY

Oncogenic driver mutations of *EGFR* and *KRAS* play a decisive role in tumor development and are biomarkers and potential therapeutic targets in lung adenocarcinoma. However, the clinical consequence of subtypes of these mutations is far less understood.

Altogether 1,247 lung adenocarcinoma patients with *KRAS* and/or *EGFR* mutation status were included in three studies. The correlations between mutations and clinicopathological data were analyzed. The therapeutic effect of platinum-based chemotherapy and EGFR-TKI treatment was evaluated in advanced or metastatic stage patients.

We have shown that the G12V subtype of *KRAS* mutation was more often present in never-smokers and conferred increased ORR and PFS in a cohort of 505 advanced-stage platinum-doublet chemotherapy treated patients. In a cohort of 814 patients with molecular analysis for potential EGFR-TKI treatment, we demonstrated that the majority of rare *EGFR* mutations were associated with smoking, shorter OS and decreased ORR to EGFR-TKI therapy when compared to classic *EGFR* mutations. The metastatic site-specific incidence of *KRAS* mutation was analyzed in a cohort of 500 adenocarcinoma patients presenting with metastatic spread at diagnosis. We have shown that intrapulmonary metastatic cases demonstrated increased *KRAS* mutation frequency when compared to extrapulmonary metastases. In contrast, pleural dissemination and liver metastasis associated with decreased mutation incidence. We found a significant negative prognostic effect of *KRAS* mutation in patients with bone spread. However, we did not found in vitro decreased ZA – a treatment frequently used in bone metastatic patients - sensitivity of KRAS mutant when compared to KRAS wild-type lung adenocarcinoma cell lines.

In summary, we demonstrated that subtype-specific molecular analysis can identify clinically relevant subgroups of patients that ultimately may influence treatment decisions. Studies focusing on oncogenic driver subtypes will further support the introduction of precision medicine into the challenging and dynamically emerging field of thoracic oncology.

8. ÖSSZEFOGLALÁS

Az *EGFR* és *KRAS* onkogén mutációi kulcsszerepet játszanak a tüdő adenocarcinomák onkogenezisében, és fontos potenciális biomarkerek, valamint terápiás célpontok is lehetnek. Ezen onkogének szubtípus specifikus mutációinak klinikai jelentősége azonban kevéssé ismert.

Vizsgálatunkban 1247, tüdő adenocarcinoma miatt kezelt, *EGFR* és/vagy *KRAS* mutációs analízissel rendelkező beteg adatait három kohorszra bontva elemeztük és vetettük össze klinikopatológiai jellemzőikkel. A terápiás hatást platina bázisú és EGFR tirozinkináz inhibitor (TKI) kezelés esetében értékeltük.

Platinabázisú kemoterápiával kezelt 505 beteg adatainak elemzése során kimutattuk, hogy a nemdohányzók aránya szignifikánsan magasabb a G12V *KRAS* mutációt hordozó betegekben, összevetve a többi *KRAS* szubtípussal. A platina alapú kezelés alkalmazásakor a G12V *KRAS* mutációt hordozóknál a terápiás válasz, és a progressziómentes túlélés is hosszabb, összehasonlítva a többi *KRAS* szubtípussal.

Az esetleges TKI terápia miatt molekuláris analízissel rendelkező 814 betegnél a ritka *EGFR* mutációk többségét a dohányzással asszociáltnak találtuk, szemben a klasszikus mutáns daganatokkal. A klasszikus *EGFR* mutációt hordozó betegek szignifikánsan jobb terápiás választ adtak TKI kezelésre, és a medián progressziómentes túlélésük is hosszabbnak bizonyult a ritka *EGFR* mutáns daganatban szenvedőkhöz képest.

A diagnóziskor már metasztázist adott tüdő adenocarcinomában szenvedő 500 beteg esetében a *KRAS* mutáció és az áttét lokalizációja közti összefüggést vizsgáltuk. A *KRAS* mutációk aránya tüdőáttéteket adó daganatokban magasabb, a mellhártya, és a májáttét jelenléte esetén pedig alacsonyabb százalékban volt jelen. Vizsgálatunk megállapította, hogy csontmetasztázist adó daganatokban a *KRAS* mutáció jelenléte rossz prognosztikus faktor. In vitro klonogenitás vizsgálat alapján zoledronsav kezelés hatása független a tüdő adenocarcinoma sejtek KRAS mutációs státuszától.

Adataink felhívják a figyelmet tüdő adenocarcinomában a szubtípus specifikus driver onkogének klinikai jelentőségére. Az onkogén mutációk pontosabb ismerete és a szubtípus mutációk kimutatása segíthet kiválasztani az elérhető leghatékonyabb terápiát az adott beteg számára.

9. REFERENCES

1. Siegel RL, Miller KD, Jemal A. (2015) Cancer statistics, 2015. CA Cancer J Clin. 65(1): p. 5-29.
2. Potosky AL, Kessler L, Gridley G, Brown CC, Horm JW. (1990) Rise in prostatic cancer incidence associated with increased use of transurethral resection. J Natl Cancer Inst. 82(20): p. 1624-8.
3. Holford TR, Cronin KA, Mariotto AB, Feuer EJ. (2006) Changing patterns in breast cancer incidence trends. J Natl Cancer Inst Monogr(36): p. 19-25.
4. Jemal A, Thun MJ, Ries LA, Howe HL, Weir HK, Center MM, Ward E, Wu XC, Eheman C, Anderson R, Ajani UA, Kohler B, Edwards BK. (2008) Annual report to the nation on the status of cancer, 1975-2005, featuring trends in lung cancer, tobacco use, and tobacco control. J Natl Cancer Inst. 100(23): p. 1672-94.
5. Pinsky PF, Church TR, Izmirlian G, Kramer BS. (2013) The National Lung Screening Trial: results stratified by demographics, smoking history, and lung cancer histology. Cancer. 119(22): p. 3976-83.
6. Ferlay J SI, Ervik M. (2013) Cancer Incidence and Mortality Worldwide, International Agency for Research on Cancer, available from: http://globocan.iarc.fr, accessed on 13th December 2013.
7. Ostoros G. (2015) A pulmonológiai hálózat 2013. évi epidemiológiai és működési adatai. Korányi Bulletin 2015(1): 36-45.
8. Zhou W, Christiani DC. (2011) East meets West: ethnic differences in epidemiology and clinical behaviors of lung cancer between East Asians and Caucasians. Chin J Cancer. 30(5): p. 287-92.
9. Moldvay J, Rokszin G, Abonyi-Toth Z, Katona L, Fabian K, Kovacs G. (2015) Lung cancer drug therapy in Hungary - 3-year experience. Onco Targets Ther. 8: p. 1031-8.
10. West L, Vidwans SJ, Campbell NP, Shrager J, Simon GR, Bueno R, Dennis PA, Otterson GA, Salgia R. (2012) A novel classification of lung cancer into molecular subtypes. PLoS One. 7(2): p. e31906.
11. Sunaga N, Shames DS, Girard L, Peyton M, Larsen JE, Imai H, Soh J, Sato M, Yanagitani N, Kaira K, Xie Y, Gazdar AF, Mori M, Minna JD. (2011) Knockdown of oncogenic KRAS in non-small cell lung cancers suppresses tumor growth and sensitizes tumor cells to targeted therapy. Mol Cancer Ther. 10(2): p. 336-46.
12. Garassino MC, Marabese M, Rusconi P, Rulli E, Martelli O, Farina G, Scanni A, Broggini M. (2011) Different types of K-Ras mutations could affect drug sensitivity and tumour behaviour in non-small-cell lung cancer. Ann Oncol. 22(1): p. 235-7.
13. Shepherd FA, Domerg C, Hainaut P, Janne PA, Pignon JP, Graziano S, Douillard JY, Brambilla E, Le Chevalier T, Seymour L, Bourredjem A, Teuff GL, Pirker R, Filipits M, Rosell R, Kratzke R, Bandarchi B, Ma X, Capelletti M, Soria JC, Tsao MS. (2013) Pooled Analysis of the Prognostic and Predictive Effects of KRAS Mutation Status and KRAS Mutation Subtype in Early-Stage Resected Non-Small-Cell Lung Cancer in Four Trials of Adjuvant Chemotherapy. J Clin Oncol. 31(17): p. 2173-81.

14. Tam IY, Chung LP, Suen WS, Wang E, Wong MC, Ho KK, Lam WK, Chiu SW, Girard L, Minna JD, Gazdar AF, Wong MP. (2006) Distinct epidermal growth factor receptor and KRAS mutation patterns in non-small cell lung cancer patients with different tobacco exposure and clinicopathologic features. Clin Cancer Res. 12(5): p. 1647-53.
15. Lovly, C., L. Horn, W. Pao. (2015) Molecular Profiling of Lung Cancer. My Cancer Genomehttp://www.mycancergenome.org/content/disease/lung-cancer/ (Updated June 17, 2015).
16. Bergethon K, Shaw AT, Ou SH, Katayama R, Lovly CM, McDonald NT, Massion PP, Siwak-Tapp C, Gonzalez A, Fang R, Mark EJ, Batten JM, Chen H, Wilner KD, Kwak EL, Clark JW, Carbone DP, Ji H, Engelman JA, Mino-Kenudson M, Pao W, Iafrate AJ. (2012) ROS1 rearrangements define a unique molecular class of lung cancers. J Clin Oncol. 30(8): p. 863-70.
17. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. (2004) Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res. 64(24): p. 8919-23.
18. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H. (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 448(7153): p. 561-6.
19. Messner I, Cadeddu G, Huckenbeck W, Knowles HJ, Gabbert HE, Baldus SE, Schaefer KL. (2013) KRAS p.G13D mutations are associated with sensitivity to anti-EGFR antibody treatment in colorectal cancer cell lines. J Cancer Res Clin Oncol. 139(2): p. 201-9.
20. Cardarella S, Ogino A, Nishino M, Butaney M, Shen J, Lydon C, Yeap BY, Sholl LM, Johnson BE, Janne PA. (2013) Clinical, pathologic, and biologic features associated with BRAF mutations in non-small cell lung cancer. Clin Cancer Res. 19(16): p. 4532-40.
21. Awad MM, Oxnard GR, Jackman DM, Savukoski DO, Hall D, Shivdasani P, Heng JC, Dahlberg SE, Janne PA, Verma S, Christensen J, Hammerman PS, Sholl LM. (2016) MET Exon 14 Mutations in Non-Small-Cell Lung Cancer Are Associated With Advanced Age and Stage-Dependent MET Genomic Amplification and c-Met Overexpression. J Clin Oncol. 34(7):721-30
22. Arcila ME, Drilon A, Sylvester BE, Lovly CM, Borsu L, Reva B, Kris MG, Solit DB, Ladanyi M. (2015) MAP2K1 (MEK1) Mutations Define a Distinct Subset of Lung Adenocarcinoma Associated with Smoking. Clin Cancer Res. 21(8): p. 1935-43.
23. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 350(21): p. 2129-39.
24. Timar J, Hegedus B, Raso E. (2010) KRAS mutation testing of colorectal cancer for anti-EGFR therapy: dogmas versus evidence. Curr Cancer Drug Targets. 10(8): p. 813-23.

25. Ohashi K, Sequist LV, Arcila ME, Lovly CM, Chen X, Rudin CM, Moran T, Camidge DR, Vnencak-Jones CL, Berry L, Pan Y, Sasaki H, Engelman JA, Garon EB, Dubinett SM, Franklin WA, Riely GJ, Sos ML, Kris MG, Dias-Santagata D, Ladanyi M, Bunn PA, Jr., Pao W. (2013) Characteristics of lung cancers harboring NRAS mutations. Clin Cancer Res. 19(9): p. 2584-91.
26. Santos E, Martin-Zanca D, Reddy EP, Pierotti MA, Della Porta G, Barbacid M. (1984) Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue of the same patient. Science. 223(4637): p. 661-4.
27. Roberts PJ, Stinchcombe TE. (2013) KRAS mutation: should we test for it, and does it matter? J Clin Oncol. 31(8): p. 1112-21.
28. Martin P, Leighl NB, Tsao MS, Shepherd FA. (2013) KRAS Mutations as Prognostic and Predictive Markers in Non-Small Cell Lung Cancer. J Thorac Oncol. 8(5): p. 530-42.
29. Bos JL. (1989) ras oncogenes in human cancer: a review. Cancer Res. 49(17): p. 4682-9.
30. Smits AJ, Kummer JA, Hinrichs JW, Herder GJ, Scheidel-Jacobse KC, Jiwa NM, Ruijter TE, Nooijen PT, Looijen-Salamon MG, Ligtenberg MJ, Thunnissen FB, Heideman DA, de Weger RA, Vink A. (2012) EGFR and KRAS mutations in lung carcinomas in the Dutch population: increased EGFR mutation frequency in malignant pleural effusion of lung adenocarcinoma. Cell Oncol (Dordr). 35(3): p. 189-96.
31. Prior IA, Lewis PD, Mattos C. (2012) A comprehensive survey of Ras mutations in cancer. Cancer Res. 72(10): p. 2457-67.
32. Trahey M, McCormick F. (1987) A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science. 238(4826): p. 542-5.
33. Ihle NT, Byers LA, Kim ES, Saintigny P, Lee JJ, Blumenschein GR, Tsao A, Liu S, Larsen JE, Wang J, Diao L, Coombes KR, Chen L, Zhang S, Abdelmelek MF, Tang X, Papadimitrakopoulou V, Minna JD, Lippman SM, Hong WK, Herbst RS, Wistuba, II, Heymach JV, Powis G. (2012) Effect of KRAS oncogene substitutions on protein behavior: implications for signaling and clinical outcome. J Natl Cancer Inst. 104(3): p. 228-39.
34. Lopez-Rios F, Angulo B, Gomez B, Mair D, Martinez R, Conde E, Shieh F, Tsai J, Vaks J, Current R, Lawrence HJ, Gonzalez de Castro D. (2013) Comparison of molecular testing methods for the detection of EGFR mutations in formalin-fixed paraffin-embedded tissue specimens of non-small cell lung cancer. J Clin Pathol. 66(5): p. 381-5.
35. Young EC, Owens MM, Adebiyi I, Bedenham T, Butler R, Callaway J, Cranston T, Crosby C, Cree IA, Dutton L, Faulkes C, Faulkner C, Howard E, Knight J, Huang Y, Lavender L, Lazarou LP, Liu H, Mair D, Milano A, Sandell S, Skinner A, Wallace A, Williams M, Spivey V, Goodall J, Frampton J, Ellard S, Clinical Molecular Genetics Society Scientific S. (2013) A comparison of methods for EGFR mutation testing in non-small cell lung cancer. Diagn Mol Pathol. 22(4): p. 190-5.
36. Nishikawa T, Maemura K, Hirata I, Matsuse R, Morikawa H, Toshina K, Murano M, Hashimoto K, Nakagawa Y, Saitoh O, Uchida K, Katsu K. (2002) A simple method of detecting K-ras point mutations in stool samples for colorectal cancer

screening using one-step polymerase chain reaction/restriction fragment length polymorphism analysis. Clin Chim Acta. 318(1-2): p. 107-12.
37. van Eijk R, Licht J, Schrumpf M, Talebian Yazdi M, Ruano D, Forte GI, Nederlof PM, Veselic M, Rabe KF, Annema JT, Smit V, Morreau H, van Wezel T. (2011) Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. PLoS One. 6(3): p. e17791.
38. McCourt CM, McArt DG, Mills K, Catherwood MA, Maxwell P, Waugh DJ, Hamilton P, O'Sullivan JM, Salto-Tellez M. (2013) Validation of next generation sequencing technologies in comparison to current diagnostic gold standards for BRAF, EGFR and KRAS mutational analysis. PLoS One. 8(7): p. e69604.
39. Chin EL, da Silva C, Hegde M. (2013) Assessment of clinical analytical sensitivity and specificity of next-generation sequencing for detection of simple and complex mutations. BMC Genet. 14: p. 6.
40. Ellison G, Zhu G, Moulis A, Dearden S, Speake G, McCormack R. (2013) EGFR mutation testing in lung cancer: a review of available methods and their use for analysis of tumour tissue and cytology samples. J Clin Pathol. 66(2): p. 79-89.
41. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, Barlesi F, Kohlhaufl M, Arrieta O, Burgio MA, Fayette J, Lena H, Poddubskaya E, Gerber DE, Gettinger SN, Rudin CM, Rizvi N, Crino L, Blumenschein GR, Jr., Antonia SJ, Dorange C, Harbison CT, Graf Finckenstein F, Brahmer JR. (2015) Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. N Engl J Med. 373(17): p. 1627-39.
42. Fossella FV, DeVore R, Kerr RN, Crawford J, Natale RR, Dunphy F, Kalman L, Miller V, Lee JS, Moore M, Gandara D, Karp D, Vokes E, Kris M, Kim Y, Gamza F, Hammershaimb L. (2000) Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-Small Cell Lung Cancer Study Group. J Clin Oncol. 18(12): p. 2354-62.
43. Garon EB, Ciuleanu TE, Arrieta O, Prabhash K, Syrigos KN, Goksel T, Park K, Gorbunova V, Kowalyszyn RD, Pikiel J, Czyzewicz G, Orlov SV, Lewanski CR, Thomas M, Bidoli P, Dakhil S, Gans S, Kim JH, Grigorescu A, Karaseva N, Reck M, Cappuzzo F, Alexandris E, Sashegyi A, Yurasov S, Perol M. (2014) Ramucirumab plus docetaxel versus placebo plus docetaxel for second-line treatment of stage IV non-small-cell lung cancer after disease progression on platinum-based therapy (REVEL): a multicentre, double-blind, randomised phase 3 trial. Lancet. 384(9944): p. 665-73.
44. Friboulet L, Olaussen KA, Pignon JP, Shepherd FA, Tsao MS, Graziano S, Kratzke R, Douillard JY, Seymour L, Pirker R, Filipits M, Andre F, Solary E, Ponsonnailles F, Robin A, Stoclin A, Dorvault N, Commo F, Adam J, Vanhecke E, Saulnier P, Thomale J, Le Chevalier T, Dunant A, Rousseau V, Le Teuff G, Brambilla E, Soria JC. (2013) ERCC1 isoform expression and DNA repair in non-small-cell lung cancer. N Engl J Med. 368(12): p. 1101-10.
45. Mascaux C, Iannino N, Martin B, Paesmans M, Berghmans T, Dusart M, Haller A, Lothaire P, Meert AP, Noel S, Lafitte JJ, Sculier JP. (2005) The role of RAS oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. British Journal of Cancer. 92(1): p. 131-139.

46. Timar J. (2014) The clinical relevance of KRAS gene mutation in non-small-cell lung cancer. Curr Opin Oncol. 26(2): p. 138-44.
47. Abramson R. (2015). Overview of Targeted Therapies for Cancer. My Cancer Genome http://www.mycancergenome.org/content/molecular-medicine/overview-of-targeted-therapies-for-cancer/ (Updated December 11, 2015).
48. Shaw AT, Ou SH, Bang YJ, Camidge DR, Solomon BJ, Salgia R, Riely GJ, Varella-Garcia M, Shapiro GI, Costa DB, Doebele RC, Le LP, Zheng Z, Tan W, Stephenson P, Shreeve SM, Tye LM, Christensen JG, Wilner KD, Clark JW, Iafrate AJ. (2014) Crizotinib in ROS1-rearranged non-small-cell lung cancer. N Engl J Med. 371(21): p. 1963-71.
49. Shaw AT, Kim DW, Mehra R, Tan DS, Felip E, Chow LQ, Camidge DR, Vansteenkiste J, Sharma S, De Pas T, Riely GJ, Solomon BJ, Wolf J, Thomas M, Schuler M, Liu G, Santoro A, Lau YY, Goldwasser M, Boral AL, Engelman JA. (2014) Ceritinib in ALK-rearranged non-small-cell lung cancer. N Engl J Med. 370(13): p. 1189-97.
50. McKeage K. (2015) Alectinib: a review of its use in advanced ALK-rearranged non-small cell lung cancer. Drugs. 75(1): p. 75-82.
51. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R, Johnson DH. (2006) Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 355(24): p. 2542-50.
52. Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA, Murray S. (2008) Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol. 9(10): p. 962-72.
53. Mao C, Qiu LX, Liao RY, Du FB, Ding H, Yang WC, Li J, Chen Q. (2010) KRAS mutations and resistance to EGFR-TKIs treatment in patients with non-small cell lung cancer: a meta-analysis of 22 studies. Lung Cancer. 69(3): p. 272-8.
54. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P. (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res. 66(8): p. 3992-5.
55. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR. (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med. 359(17): p. 1757-65.
56. A Study of Necitumumab and Chemotherapy in Participants With Stage IV Squamous Non-Small Cell Lung Cancer [Last updated: May 8, 2015]. Available from:https://clinicaltrials.gov/ct2/show/NCT01769391 NLM Identifier: NCT01769391.
57. Khambata-Ford S, Harbison CT, Hart LL, Awad M, Xu LA, Horak CE, Dakhil S, Hermann RC, Lynch TJ, Weber MR. (2010) Analysis of potential predictive markers of cetuximab benefit in BMS099, a phase III study of cetuximab and first-line taxane/carboplatin in advanced non-small-cell lung cancer. J Clin Oncol. 28(6): p. 918-27.

58. O'Byrne KJ, Gatzemeier U, Bondarenko I, Barrios C, Eschbach C, Martens UM, Hotko Y, Kortsik C, Paz-Ares L, Pereira JR, von Pawel J, Ramlau R, Roh JK, Yu CT, Stroh C, Celik I, Schueler A, Pirker R. (2011) Molecular biomarkers in non-small-cell lung cancer: a retrospective analysis of data from the phase 3 FLEX study. Lancet Oncol. 12(8): p. 795-805.
59. Kerner GS, Schuuring E, Sietsma J, Hiltermann TJ, Pieterman RM, de Leede GP, van Putten JW, Liesker J, Renkema TE, van Hengel P, Platteel I, Timens W, Groen HJ, Consortium CAF. (2013) Common and rare EGFR and KRAS mutations in a Dutch non-small-cell lung cancer population and their clinical outcome. PLoS One. 8(7): p. e70346.
60. D'Arcangelo M, D'Incecco A, Cappuzzo F. (2013) Rare mutations in non-small-cell lung cancer. Future Oncol. 9(5): p. 699-711.
61. Yasuda H, Kobayashi S, Costa DB. (2012) EGFR exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications. Lancet Oncol. 13(1): p. e23-31.
62. Thongprasert S, Duffield E, Saijo N, Wu YL, Yang JC, Chu DT, Liao M, Chen YM, Kuo HP, Negoro S, Lam KC, Armour A, Magill P, Fukuoka M. (2011) Health-related quality-of-life in a randomized phase III first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients from Asia with advanced NSCLC (IPASS). J Thorac Oncol. 6(11): p. 1872-80.
63. Dungo RT, Keating GM. (2013) Afatinib: first global approval. Drugs. 73(13): p. 1503-15.
64. Sequist LV, Yang JC, Yamamoto N, O'Byrne K, Hirsh V, Mok T, Geater SL, Orlov S, Tsai CM, Boyer M, Su WC, Bennouna J, Kato T, Gorbunova V, Lee KH, Shah R, Massey D, Zazulina V, Shahidi M, Schuler M. (2013) Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. J Clin Oncol. 31(27): p. 3327-34.
65. Rosell R, Molina MA, Costa C, Simonetti S, Gimenez-Capitan A, Bertran-Alamillo J, Mayo C, Moran T, Mendez P, Cardenal F, Isla D, Provencio M, Cobo M, Insa A, Garcia-Campelo R, Reguart N, Majem M, Viteri S, Carcereny E, Porta R, Massuti B, Queralt C, de Aguirre I, Sanchez JM, Sanchez-Ronco M, Mate JL, Ariza A, Benlloch S, Sanchez JJ, Bivona TG, Sawyers CL, Taron M. (2011) Pretreatment EGFR T790M mutation and BRCA1 mRNA expression in erlotinib-treated advanced non-small-cell lung cancer patients with EGFR mutations. Clin Cancer Res. 17(5): p. 1160-8.
66. Boch C, Kollmeier J, Roth A, Stephan-Falkenau S, Misch D, Gruning W, Bauer TT, Mairinger T. (2013) The frequency of EGFR and KRAS mutations in non-small cell lung cancer (NSCLC): routine screening data for central Europe from a cohort study. BMJ Open. 3(4) 3:e002560.
67. Carrizosa DR, Gold KA. (2015) New strategies in immunotherapy for non-small cell lung cancer. Transl Lung Cancer Res. 4(5): p. 553-9.
68. Villadolid J, Amin A. (2015) Immune checkpoint inhibitors in clinical practice: update on management of immune-related toxicities. Transl Lung Cancer Res. 4(5): p. 560-75.
69. Torok S, Hegedus B, Laszlo V, Hoda MA, Ghanim B, Berger W, Klepetko W, Dome B, Ostoros G. (2011) Lung cancer in never smokers. Future Oncol. 7(10): p. 1195-211.

70. Nordquist LT, Simon GR, Cantor A, Alberts WM, Bepler G. (2004) Improved survival in never-smokers vs current smokers with primary adenocarcinoma of the lung. Chest. 126(2): p. 347-51.
71. Toh CK, Wong EH, Lim WT, Leong SS, Fong KW, Wee J, Tan EH. (2004) The impact of smoking status on the behavior and survival outcome of patients with advanced non-small cell lung cancer: a retrospective analysis. Chest. 126(6): p. 1750-6.
72. Tammemagi CM, Neslund-Dudas C, Simoff M, Kvale P. (2004) Smoking and lung cancer survival: the role of comorbidity and treatment. Chest. 125(1): p. 27-37.
73. Kim YT, Seong YW, Jung YJ, Jeon YK, Park IK, Kang CH, Kim JH. (2013) The presence of mutations in epidermal growth factor receptor gene is not a prognostic factor for long-term outcome after surgical resection of non-small-cell lung cancer. J Thorac Oncol. 8(2): p. 171-8.
74. Slebos RJ, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJ, Wagenaar SS, Vanderschueren RG, van Zandwijk N, Mooi WJ, et al. (1990) K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med. 323(9): p. 561-5.
75. Rosell R, Li S, Skacel Z, Mate JL, Maestre J, Canela M, Tolosa E, Armengol P, Barnadas A, Ariza A. (1993) Prognostic impact of mutated K-ras gene in surgically resected non-small cell lung cancer patients. Oncogene. 8(9): p. 2407-12.
76. Mitsudomi T, Steinberg SM, Oie HK, Mulshine JL, Phelps R, Viallet J, Pass H, Minna JD, Gazdar AF. (1991) ras gene mutations in non-small cell lung cancers are associated with shortened survival irrespective of treatment intent. Cancer Res. 51(18): p. 4999-5002.
77. Massarelli E, Johnson FM, Erickson HS, Wistuba, II, Papadimitrakopoulou V. (2013) Uncommon epidermal growth factor receptor mutations in non-small cell lung cancer and their mechanisms of EGFR tyrosine kinase inhibitors sensitivity and resistance. Lung Cancer. 80(3): p. 235-41.
78. Yeh P, Chen H, Andrews J, Naser R, Pao W, Horn L. (2013) DNA-Mutation Inventory to Refine and Enhance Cancer Treatment (DIRECT): a catalog of clinically relevant cancer mutations to enable genome-directed anticancer therapy. Clin Cancer Res. 19(7): p. 1894-901.
79. Mirsadraee S, Oswal D, Alizadeh Y, Caulo A, van Beek E, Jr. (2012) The 7th lung cancer TNM classification and staging system: Review of the changes and implications. World J Radiol. 4(4): p. 128-34.
80. Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R, Postmus PE, Rusch V, Sobin L, International Association for the Study of Lung Cancer International Staging C, Participating I. (2007) The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol. 2(8): p. 706-14.
81. Szabo B, Nelhubel GA, Karpati A, Kenessey I, Jori B, Szekely C, Petak I, Lotz G, Hegedus Z, Hegedus B, Fule T, Dome B, Timar J, Tovari J. (2011) Clinical significance of genetic alterations and expression of epidermal growth factor

receptor (EGFR) in head and neck squamous cell carcinomas. Oral Oncol. 47(6): p. 487-96.
82. Hegedus Z, Barbai T, Tatrai P, Hegedus B, Kiss A, Raso E, Bodoky G. (2011) [Effect of KRAS mutation status on the efficiency of Avastin therapy of colorectal cancer]. Magy Onkol. 55(2): p. 99-100, 102-4.
83. Cancer Therapy Evaluation Program NCI. Common Terminology Criteria for Adverse Events (CTCAE) v3.0 Online Instructions and Guidelines. https://webapps.ctep.nci.nih.gov/webobjs/ctc/webhelp/welcome_to_ctcae.htm. Accessed May 19, 2009.
84. Brower M, Carney DN, Oie HK, Gazdar AF, Minna JD. (1986) Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. Cancer Res. 46(2): p. 798-806.
85. Fogh J, Wright WC, Loveless JD. (1977) Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J Natl Cancer Inst. 58(2): p. 209-14.
86. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP. (1973) In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst. 51(5): p. 1417-23.
87. Sordella R, Bell DW, Haber DA, Settleman J. (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science. 305(5687): p. 1163-7.
88. Phelps RM, Johnson BE, Ihde DC, Gazdar AF, Carbone DP, McClintock PR, Linnoila RI, Matthews MJ, Bunn PA Jr, Carney D, Minna JD, Mulshine JL. (1996) *NCI-Navy Medical Oncology Branch cell line supplement.* J Cell Biochem Suppl. **24**: p. 1-291.
89. Davies KD, Le AT, Theodoro MF, Skokan MC, Aisner DL, Berge EM, Terracciano LM, Cappuzzo F, Incarbone M, Roncalli M, Alloisio M, Santoro A, Camidge DR, Varella-Garcia M, Doebele RC. (2012) *Identifying and targeting ROS1 gene fusions in non-small cell lung cancer.* Clin Cancer Res. **18**(17): p. 4570-9.
90. Wu JY, Yu CJ, Chang YC, Yang CH, Shih JY, Yang PC. (2011) *Effectiveness of tyrosine kinase inhibitors on "uncommon" epidermal growth factor receptor mutations of unknown clinical significance in non-small cell lung cancer.* Clin Cancer Res. **17**(11): p. 3812-21.
91. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal PA, Stratton MR, Wooster R. (2004) *The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website.* Br J Cancer. **91**(2): p. 355-8.
92. Ramlau R, Cufer T, Berzinec P, Dziadziuszko R, Olszewski W, Popper H, Bajcic P, Dusek L, Zbozinkova Z, Pirker R, team Is. (2015) *Epidermal Growth Factor Receptor Mutation-Positive Non-Small-Cell Lung Cancer in the Real-World Setting in Central Europe: The INSIGHT Study.* J Thorac Oncol. **10**(9): p. 1370-4.
93. Eberhard DA, Johnson BE, Amler LC, Goddard AD, Heldens SL, Herbst RS, Ince WL, Janne PA, Januario T, Johnson DH, Klein P, Miller VA, Ostland MA, Ramies DA, Sebisanovic D, Stinson JA, Zhang YR, Seshagiri S, Hillan KJ. (2005) *Mutations in the epidermal growth factor receptor and in KRAS are predictive*

and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. Journal of Clinical Oncology. **23**(25): p. 5900-5909.

94. Pallis AG, Voutsina A, Kalikaki A, Souglakos J, Briasoulis E, Murray S, Koutsopoulos A, Tripaki M, Stathopoulos E, Mavroudis D, Georgoulias V. (2007) *'Classical' but not 'other' mutations of EGFR kinase domain are associated with clinical outcome in gefitinib-treated patients with non-small cell lung cancer*. Br J Cancer. **97**(11): p. 1560-6.
95. Arcila ME, Nafa K, Chaft JE, Rekhtman N, Lau C, Reva BA, Zakowski MF, Kris MG, Ladanyi M. (2013) *EGFR Exon 20 Insertion Mutations in Lung Adenocarcinomas: Prevalence, Molecular Heterogeneity, and Clinicopathologic Characteristics.* Mol Cancer Ther. **12**(2): p. 220-229.
96. Yang JC, Shih JY, Su WC, Hsia TC, Tsai CM, Ou SH, Yu CJ, Chang GC, Ho CL, Sequist LV, Dudek AZ, Shahidi M, Cong XJ, Lorence RM, Yang PC, Miller VA. (2012) *Afatinib for patients with lung adenocarcinoma and epidermal growth factor receptor mutations (LUX-Lung 2): a phase 2 trial.* Lancet Oncol. **13**(5): p. 539-48.
97. Keam B, Kim DW, Park JH, Lee JO, Kim TM, Lee SH, Jeon YK, Chung DH, Heo DS. (2012) *Rare and complex mutations of epidermal growth factor receptor (EGFR) and efficacy of tyrosine kinase inhibitor (TKI) in patients with non-small cell lung cancer (NSCLC).* Journal of Clinical Oncology. **30**(15): p. 594-600.
98. de Mello RA, Pires FS, Marques DS, Oliveira J, Rodrigues A, Soares M, Azevedo I, Peixoto A, Santos C, Pinto C, Hespanhol V, Teixeira MR, Amaro T, Queiroga H, Araujo A. (2012) *EGFR exon mutation distribution and outcome in non-small-cell lung cancer: a Portuguese retrospective study.* Tumour Biol. **33**(6): p. 2061-8.
99. Sandelin M, Berglund A, Sundstrom M, Micke P, Ekman S, Bergqvist M, Bergstrom S, Koyi H, Branden E, Janson C, Botling J. (2015) *Patients with Non-small Cell Lung Cancer Analyzed for EGFR: Adherence to Guidelines, Prevalence and Outcome.* Anticancer Res. **35**(7): p. 3979-85.
100. Errihani H, Inrhaoun H, Boukir A, Kettani F, Gamra L, Mestari A, Jabri L, Bensouda Y, Mrabti H, Elghissassi I. (2013) *Frequency and type of epidermal growth factor receptor mutations in moroccan patients with lung adenocarcinoma.* J Thorac Oncol. **8**(9): p. 1212-4.
101. Elghissassi I, Errihani H. (2013) *Spectrum of EGFR mutation in lung adenocarcinoma in Morocco.* J Thorac Oncol. **8**(12): p. e115-6.
102. De Pas T, Toffalorio F, Manzotti M, Fumagalli C, Spitaleri G, Catania C, Delmonte A, Giovannini M, Spaggiari L, de Braud F, Barberis M. (2011) *Activity of epidermal growth factor receptor-tyrosine kinase inhibitors in patients with non-small cell lung cancer harboring rare epidermal growth factor receptor mutations.* J Thorac Oncol. **6**(11): p. 1895-901.
103. Costa C, Molina-Vila MA, Drozdowskyj A, Gimenez-Capitan A, Bertran-Alamillo J, Karachaliou N, Gervais R, Massuti B, Wei J, Moran T, Majem M, Felip E, Carcereny E, Garcia-Campelo R, Viteri S, Taron M, Ono M, Giannikopolous P, Bivona TG, Rosell R. (2014) *The impact of EGFR T790M mutations and BIM mRNA expression on outcome in patients with EGFR-mutant*

NSCLC treated with erlotinib or chemotherapy in the randomized phase III EURTAC trial. Clin Cancer Res. **20**(7):2001-10.

104. Beau-Faller M, Prim N, Ruppert AM, Nanni-Metellus I, Lacave R, Lacroix L, Escande F, Lizard S, Pretet JL, Rouquette I, de Cremoux P, Solassol J, de Fraipont F, Bieche I, Cayre A, Favre-Guillevin E, Tomasini P, Wislez M, Besse B, Legrain M, Voegeli AC, Baudrin L, Morin F, Zalcman G, Quoix E, Blons H, Cadranel J. (2014) *Rare EGFR exon 18 and exon 20 mutations in non-small-cell lung cancer on 10 117 patients: a multicentre observational study by the French ERMETIC-IFCT network.* Annals of Oncology. **25**(1): p. 126-31.
105. Riely GJ, Kris MG, Rosenbaum D, Marks J, Li A, Chitale DA, Nafa K, Riedel ER, Hsu M, Pao W, Miller VA, Ladanyi M. (2008) *Frequency and distinctive spectrum of KRAS mutations in never smokers with lung adenocarcinoma.* Clin Cancer Res. **14**(18): p. 5731-4.
106. Husgafvel-Pursiainen K, Hackman P, Ridanpaa M, Anttila S, Karjalainen A, Partanen T, Taikina-Aho O, Heikkila L, Vainio H. (1993) *K-ras mutations in human adenocarcinoma of the lung: association with smoking and occupational exposure to asbestos.* Int J Cancer. **53**(2): p. 250-6.
107. Kim HR, Ahn JR, Lee JG, Bang DH, Ha SJ, Hong YK, Kim SM, Nam KC, Rha SY, Soo RA, Riely GJ, Kim JH, Cho BC. (2013) *The Impact of Cigarette Smoking on the Frequency of and Qualitative Differences in KRAS Mutations in Korean Patients with Lung Adenocarcinoma.* Yonsei Med J. **54**(4): p. 865-74.
108. Sun JM, Hwang DW, Ahn JS, Ahn MJ, Park K. (2013) *Prognostic and Predictive Value of KRAS Mutations in Advanced Non-Small Cell Lung Cancer.* PLoS One. **8**(5): p. e64816.
109. Yu HA, Sima CS, Shen R, Kass SL, Kris MG, Ladanyi M, Riely GJ. (2013) *Comparison of the characteristics and clinical course of 677 patients with metastatic lung cancers with mutations in KRAS codons 12 and 13.* J Clin Oncol **31**(suppl; abstr 8025).
110. Tsai TH, Su KY, Wu SG, Chang YL, Luo SC, Jan IS, Yu CJ, Yu SL, Shih JY, Yang PC. (2012) *RNA is favourable for analysing EGFR mutations in malignant pleural effusion of lung cancer.* Eur Respir J. **39**(3): p. 677-84.
111. Hofreiter M, Jaenicke V, Serre D, von Haeseler A, Paabo S. (2001) *DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA.* Nucleic Acids Res. **29**(23): p. 4793-9.
112. Qiu M, Wang J, Xu Y, Ding X, Li M, Jiang F, Xu L, Yin R. (2015) *Circulating tumor DNA is effective for the detection of EGFR mutation in non-small cell lung cancer: a meta-analysis.* Cancer Epidemiol Biomarkers Prev. **24**(1): p. 206-12.
113. Johnson ML, Sima CS, Chaft J, Paik PK, Pao W, Kris MG, Ladanyi M, Riely GJ. (2013) *Association of KRAS and EGFR mutations with survival in patients with advanced lung adenocarcinomas.* Cancer. **119**(2): p. 356-62.
114. Mellema WW, Dingemans AM, Thunnissen E, Snijders PJ, Derks J, Heideman DA, Van Suylen R, Smit EF. (2013) *KRAS mutations in advanced nonsquamous non-small-cell lung cancer patients treated with first-line platinum-based chemotherapy have no predictive value.* J Thorac Oncol. **8**(9): p. 1190-5.
115. Rodenhuis S, Boerrigter L, Top B, Slebos RJ, Mooi WJ, van't Veer L, van Zandwijk N. (1997) *Mutational activation of the K-ras oncogene and the effect of*

chemotherapy in advanced adenocarcinoma of the lung: a prospective study. Journal of Clinical Oncology. **15**(1): p. 285-91.

116. Villaruz LC, Socinski MA, Cunningham DE, Chiosea SI, Burns TF, Siegfried JM, Dacic S. (2013) *The prognostic and predictive value of KRAS oncogene substitutions in lung adenocarcinoma.* Cancer. **119**(12): p. 2268-74.
117. Tsao MS, Aviel-Ronen S, Ding K, Lau D, Liu N, Sakurada A, Whitehead M, Zhu CQ, Livingston R, Johnson DH, Rigas J, Seymour L, Winton T, Shepherd FA. (2007) *Prognostic and predictive importance of p53 and RAS for adjuvant chemotherapy in non small-cell lung cancer.* Journal of Clinical Oncology. **25**(33): p. 5240-7.
118. Renaud S, Falcoz PE, Schaeffer M, Guenot D, Romain B, Olland A, Reeb J, Santelmo N, Chenard MP, Legrain M, Voegeli AC, Beau-Faller M, Massard G. (2015) *Prognostic value of the KRAS G12V mutation in 841 surgically resected Caucasian lung adenocarcinoma cases.* Br J Cancer. **113**(8): p. 1206-15.
119. Shepherd FA, Domerg C, Hainaut P, Janne PA, Pignon JP, Graziano S, Douillard JY, Brambilla E, Le Chevalier T, Seymour L, Bourredjem A, Le Teuff G, Pirker R, Filipits M, Rosell R, Kratzke R, Bandarchi B, Ma X, Capelletti M, Soria JC, Tsao MS. (2013) *Pooled analysis of the prognostic and predictive effects of KRAS mutation status and KRAS mutation subtype in early-stage resected non-small-cell lung cancer in four trials of adjuvant chemotherapy.* J Clin Oncol. **31**(17): p. 2173-81.
120. Imamura Y, Morikawa T, Liao X, Lochhead P, Kuchiba A, Yamauchi M, Qian ZR, Nishihara R, Meyerhardt JA, Haigis KM, Fuchs CS, Ogino S. (2012) *Specific mutations in KRAS codons 12 and 13, and patient prognosis in 1075 BRAF wild-type colorectal cancers.* Clin Cancer Res. **18**(17): p. 4753-63.
121. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, Palmero R, Garcia-Gomez R, Pallares C, Sanchez JM, Porta R, Cobo M, Garrido P, Longo F, Moran T, Insa A, De Marinis F, Corre R, Bover I, Illiano A, Dansin E, de Castro J, Milella M, Reguart N, Altavilla G, Jimenez U, Provencio M, Moreno MA, Terrasa J, Munoz-Langa J, Valdivia J, Isla D, Domine M, Molinier O, Mazieres J, Baize N, Garcia-Campelo R, Robinet G, Rodriguez-Abreu D, Lopez-Vivanco G, Gebbia V, Ferrera-Delgado L, Bombaron P, Bernabe R, Bearz A, Artal A, Cortesi E, Rolfo C, Sanchez-Ronco M, Drozdowskyj A, Queralt C, de Aguirre I, Ramirez JL, Sanchez JJ, Molina MA, Taron M, Paz-Ares L, Pneumocancerologie GF, Toracica AIO. (2012) *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 Oncology. **13**(3): p. 239-246.

122. Douillard JY, Ostoros G, Cobo M, Ciuleanu T, McCormack R, Webster A, Milenkova T. (2014) *First-line gefitinib in Caucasian EGFR mutation-positive NSCLC patients: a phase-IV, open-label, single-arm study.* Br J Cancer. **110**(1): p. 55-62.
123. Doebele RC, Lu X, Sumey C, Maxson DA, Weickhardt AJ, Oton AB, Bunn PA, Jr., Baron AE, Franklin WA, Aisner DL, Varella-Garcia M, Camidge DR. (2012) *Oncogene status predicts patterns of metastatic spread in treatment-naive nonsmall cell lung cancer.* Cancer. **118**(18): p. 4502-11.

124. Modest DP, Stintzing S, Laubender RP, Neumann J, Jung A, Giessen C, Haas M, Aubele P, Schulz C, Boeck S, Stemmler HJ, Kirchner T, Heinemann V. (2011) *Clinical characterization of patients with metastatic colorectal cancer depending on the KRAS status.* Anticancer Drugs. **22**(9): p. 913-8.
125. Confavreux CB, Girard N, Pialat JB, Bringuier PP, Devouassoux-Shisheboran M, Rousseau JC, Isaac S, Thivolet-Bejui F, Clezardin P, Brevet M. (2014) *Mutational profiling of bone metastases from lung adenocarcinoma: results of a prospective study (POUMOS-TEC).* Bonekey Rep. **3**: p. 580.
126. Bittner N, Baliko Z, Sarosi V, Laszlo T, Toth E, Kasler M, Geczi L. (2015) *Bone Metastases and the EGFR and KRAS Mutation Status in Lung Adenocarcinoma--The Results of Three Year Retrospective Analysis.* Pathol Oncol Res. **21**(4): p. 1217-21.
127. Schweiger T, Hegedus B, Nikolowsky C, Hegedus Z, Szirtes I, Mair R, Birner P, Dome B, Lang G, Klepetko W, Ankersmit HJ, Hoetzenecker K. (2014) *EGFR, BRAF and KRAS status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study.* Ann Surg Oncol. **21**(3): p. 946-54.
128. Yaeger R, Cowell E, Chou JF, Gewirtz AN, Borsu L, Vakiani E, Solit DB, Rosen N, Capanu M, Ladanyi M, Kemeny N. (2015) *RAS mutations affect pattern of metastatic spread and increase propensity for brain metastasis in colorectal cancer.* Cancer. **121**(8): p. 1195-203.
129. Tsunoda A, Iijima T, Tsunoda Y, Nakao K, Miyaki M, Kusano M. (2004) *Association of K-ras mutations with liver metastases from colorectal carcinoma.* Anticancer Res. **24**(4): p. 2471-6.
130. He YY, Zhang XC, Yang JJ, Niu FY, Zeng Z, Yan HH, Xu CR, Guan JL, Zhong WZ, Yang LL, Guo LH, Wu YL. (2014) *Prognostic significance of genotype and number of metastatic sites in advanced non-small-cell lung cancer.* Clin Lung Cancer. **15**(6): p. 441-7.
131. Eberhardt WE, Mitchell A, Crowley J, Kondo H, Kim YT, Turrisi A, 3rd, Goldstraw P, Rami-Porta R, International Association for the Study of Lung Cancer S, Prognostic Factors Committee ABM, Participating I. (2015) *The IASLC Lung Cancer Staging Project: Proposals for the Revision of the M Descriptors in the Forthcoming Eighth Edition of the TNM Classification of Lung Cancer.* J Thorac Oncol. **10**(11): p. 1515-22.
132. Langer CJ, Mehta MP. (2005) *Current management of brain metastases, with a focus on systemic options.* J Clin Oncol. **23**(25): p. 6207-19.
133. Kepka L, Cieslak E, Bujko K, Fijuth J, Wierzchowski M. (2005) *Results of the whole-brain radiotherapy for patients with brain metastases from lung cancer: the RTOG RPA intra-classes analysis.* Acta Oncol. **44**(4): p. 389-98.
134. Watanabe K, Saito N, Sugito M, Ito M, Kobayashi A, Nishizawa Y. (2013) *Incidence and predictive factors for pulmonary metastases after curative resection of colon cancer.* Ann Surg Oncol. **20**(4): p. 1374-80.
135. Watanabe K, Saito N, Sugito M, Ito M, Kobayashi A, Nishizawa Y. (2011) *Predictive factors for pulmonary metastases after curative resection of rectal cancer without preoperative chemoradiotherapy.* Dis Colon Rectum. **54**(8): p. 989-98.

136. Varol Y, Varol U, Karaca B, Karabulut B, Sezgin C, Uslu R. (2012) *The frequency and significance of radiologically detected indeterminate pulmonary nodules in patients with colorectal cancer.* Med Princ Pract. **21**(5): p. 457-61.
137. Murin S, Inciardi J. (2001) *Cigarette smoking and the risk of pulmonary metastasis from breast cancer.* Chest. **119**(6): p. 1635-40.
138. Abrams JA, Lee PC, Port JL, Altorki NK, Neugut AI. (2008) *Cigarette smoking and risk of lung metastasis from esophageal cancer.* Cancer Epidemiol Biomarkers Prev. **17**(10): p. 2707-13.
139. Garay T, Kenessey I, Molnar E, Juhasz E, Reti A, Laszlo V, Rozsas A, Dobos J, Dome B, Berger W, Klepetko W, Tovari J, Timar J, Hegedus B. (2015) *Prenylation inhibition-induced cell death in melanoma: reduced sensitivity in BRAF mutant/PTEN wild-type melanoma cells.* PLoS One. **10**(2): p. e0117021.

10. LIST OF PUBLICATIONS

10.1. Publications related to the thesis

1. Hegedűs B, Moldvay J, Berta J, **Lohinai Z**, Rózsás A, Cserepes MT, Fábián K, Ostoros G, Tóvári J, Rényi-Vámos F, Tímár J, Döme B. [Excerpts from the collaborative lung cancer research program of Semmelweis University, the National Institute of Oncology and Korányi Institute of TB and Pulmonology (2010-2015)]. Magy Onkol. 2015 Dec;59(4):282-5. Hungarian.

2. Lohinai Z, Hoda MA, Fabian K, Ostoros G, Raso E, Barbai T, Timar J, Kovalszky Cserepes M, Rozsas A, Laszlo V, Grusch M, Berger W, Klepetko W, Moldvay J,Dome B, Hegedus B. Distinct Epidemiology and Clinical Consequence of Classic Versus Rare *EGFR* Mutations in Lung Adenocarcinoma. J Thorac Oncol. 2015 May;10(5):738-46. (IF: 5.28),

Commentar:

1. **Lohinai Z**, Ostoros G, Moldvay J, Dome B, Hegedus B. Reply to Rare Versus Artifactual *EGFR* Mutations. J Thorac Oncol. 2015 Aug;10(8):e80-1.

2. **Lohinai Z**, Ostoros G, Moldvay J, Dome B, Hegedus B. Differences in the Epidemiology of Rare *EGFR* Mutations in Different Populations. J Thorac Oncol. 2016 Jan;11(1):e19-20.

3. Cserepes M, Ostoros G, **Lohinai Z**, Raso E, Barbai T, Timar J, Rozsas A, Moldvay J, Kovalszky I, Fabian K, Gyulai M, Ghanim B, Laszlo V, Klikovits T, Hoda MA,Grusch M, Berger W, Klepetko W, Hegedus B, Dome B. Subtype-specific *KRAS* mutations in advanced lung adenocarcinoma: a retrospective study of patients treated with platinum-based chemotherapy. Eur J Cancer. 2014 Jul;50(10):1819-28 (IF: 5.41),

4. **Lohinai Z**, Ostoros Gy, Cserepes TM, Rásó E, Tímár J, Döme B, Hegedűs B Az EGFR mutáció epidemiológiája tüdő adenocarcinomában: hazai tapasztalatok. Medicina Thoracalis (Budapest) 2013 Aug 66:(4) pp. 211-217.

10.2. Publications not related to the thesis

1. **Lohinai Z**, Peter Dome, Zsuzsa Szilagyi, Gyula Ostoros, Judit Moldvay, Balazs Hegedus, Balazs Dome, Glen J. Weiss From Bench to Bedside: Attempt to Validate Repositioning of Drugs in the Treatment of Metastatic Small Cell Lung Cancer (SCLC). PLoS One. 2016 Jan 6;11(1):e0144797 (IF: 3.23)

2. Maneschg OA, Volek É, **Lohinai Z**, Resch MD, Papp A, Korom Cs, Karlinger K, Németh J. Genauigkeit und Relevanz der CT Volumetrie bei offenen Bulbusverletzungen mit Intraokularen Fremdkörpern. Ophthalmologe 2015 Apr; 112(4):367. (IF: 0.504)

3. Ostoros Gyula**, Lohinai Zoltán** Új lehetőségek a nem kissejtes tüdőrák másodvonalbeli kezelésében Onkológia (ISSN: 2062-7041) 2014. 4. évf.: (2. sz.,) pp. 93-95.

11. ACKNOWLEDGEMENTS

During my PhD studies, I had a great opportunity to meet many helpful people in a number of different hospitals institutions and laboratories. First, I am grateful to my home Institute, the National Korányi Institute of TB and Pulmonology, Budapest. It has been a great opportunity to perform research at the Comprehensive Cancer Center and Translational Thoracic Oncology Laboratory at the Medical University of Vienna, Cancer Treatment Centers of America and the University of Colorado in the US.

My first debt of appreciation goes to my advisors, Dr. Balázs Döme and Dr. Balázs Hegedűs, who guided and managed me during my research. Special acknowledgements also go to Dr. Gábor Kovács, the Head of the Institution who supported my projects and scientific career.

Special thanks are extended to Dr. József Tímár, Dr. Judit Moldvay, Dr. Walter Klepetko, Dr. Robert Pirker, Dr. Glen J. Weiss, Dr. Gyula Ostoros, Dr. Paul Bunn, Dr. Zsolt Markóczy, Dr Tamás Garay and the staff of the participating laboratories and institutions.

Also, I am grateful to my family for supporting me during my PhD studies.

12. APPENDIX

Supplemental Table 1. List of rare and synonymous *EGFR* mutations in cohort 1 (n=77 patients, N=98 mutations were identified).

Patient ID	Sign of the aminoacid change	Gender	Age (diagnosis)	Smoking status	Stage at diagnosis	Drug	TKI line	Response	Censored	PFS	Reference PMID	COSMIC ID	Tissue distribution
1	V845V	f	64,0	current	IA	E	3	PD	y	3,4	novel	-	-
2	P848P	f	66,0	current	IV	E	2	PR	n	5,1	novel	-	-
2	frameshift *†	f	66,0	current	IV	E	2	PR	n	5,1	novel	-	-
3	K713K	m	65,4	former	IIIA	E	3	PD	y	3	novel	-	-
4	K708K	f	43,1	former	IIIA	E	2	SD	y	14,7	-	COSM1451573	large intestine
5	G724A	f	61,3	former	IIB	E	2	PD	y	2,8	novel	-	-
6	E709-T710 del	m	69,2	former	IV	E	2	SD	y	6	novel	-	-
7	L788L	f	64,9	never	IIIA	E	3	PR	n	19,2	22980975	COSM366483	lung
7	F795C	f	64,9	never	IIIA	E	3	PR	n	19,2	novel	-	-
7	18, del30	f	64,9	never	IIIA	E	3	PR	n	19,2	novel	-	-
8	V851I	f	65,7	never	IV	E	1	PR	n	15,5	23485129	COSM12727	lung
9	E709A	m	74,4	current	IIB	E	2	N/A	y	0	23344264	COSM13427	lung
10	K716R	f	66,6	current	IIIA	E	2	PD	y	1,2	19020901	COSM28512	lung
10	I744V	f	66,6	current	IIIA	E	2	PD	y	1,2	23344264	COSM28512	lung
11	G719S	m	67,4	current	IV	E	3	N/A	n	3,4	18176089	COSM6252	lung
12	G719S	m	55,1	current	IB	E	2	SD	n	2,1	23344264	COSM6252	lung
13	R836R	m	N/A	current	N/A	E	2	PR	n	3,5	21274259	COSM85893	upper aerodigestive tract
14	G796S	m	56,5	former	IV	E	2	SD	y	4,7	18528899	COSM20891	head and neck
14	C818Y	m	56,5	former	IV	E	2	SD	y	4,7	17354229	COSM23611	pancreas
15	V845M	f	75,7	never	IIA	E	2	N/A	n	N/A	19190079	COSM28931	adrenal
16	L799L	f	76,3	never	IV	E	2	SD	y	6,2	16467085	-	lung
17	P794P	f	75,0	former	IB	G	1	PD	y	2,2	novel	-	-
18	L852R	f	71,9	never	IV	G	1	PR	n	19	novel	-	-
19	L852R	f	73,4	never	N/A	G	1	PR	y	7	novel	-	-
20	C797S	f	75,2	never	IIIA	G	1	SD	y	6,6	novel	-	-
21	P848L	m	57,2	current	IV	G	1	SD	n	1,7	23897956	COSM22943	lung
22	E709_T710>D	f	77,0	never	IB	G	1	PR	y	6,2	20559149	COSM51525	lung
23	L861Q	f	63,9	never	IIIB	N/A	N/A	PR	N/A	N/A	23344264	COSM6213	lung
24	G719A	m	53,8	never	IV	G	1	PR	n	7	23344265	COSM6240	lung
25	G719A	m	57,8	current	IV	G	1	SD	n	7,8	23344266	COSM6241	lung
26	T854I*	m	57,5	former	IA	G	1	PR	n	18	21409490	COSM87246	lung
26	T785I*	m	57,5	former	IA	G	1	PR	n	18	23139256	COSM53288	lung
27	P753P	m	52,2	former	IV	G	1	PR	n	1,5	16707764	COSM18489	oesophagus
27	I759V	m	52,2	former	IV	G	1	PR	n	1,5	19776290	COSM36938	thyroid gland
28	E734D	f	72,0	current	IIIB	G	1	PD	y	2,1	-	COSM1451582	large intestine
29	M825I	f	74,7	current	IV	G	1	PR	y	2,5	2329544	COSM1578128	biliary tract
30	A839V	m	69,2	never	IB	G	1	PD	y	3,5	21266046	COSM85900	prostate
31	V802V	m	67,8	former	IA	x	x	x	x	x	novel	-	-
31	T854I	m	67,8	former	IA	x	x	x	x	x	21409490	COSM87246	lung
31	V851I	m	67,8	former	IA	x	x	x	x	x	23485129	COSM12727	lung
32	L799L	f	55,5	current	IIIB	x	x	x	x	x	novel	-	-
32	V786V	f	55,5	current	IIIB	x	x	x	x	x	novel	-	-
32	G779D	f	55,5	current	IIIB	x	x	x	x	x	21057810	COSM133202	lung
32	G768N	f	55,5	current	IIIB	x	x	x	x	x	23139256	COSM14068	lung
33	V744V	m	75,1	former	IA	x	x	x	x	x	novel	-	-
34	L838L	f	54,7	never	IA	x	x	x	x	x	16203769	-	lung
34	T783T	f	54,7	never	IA	x	x	x	x	x	novel	-	-
35	S784S	m	55,6	current	IIIA	x	x	x	x	x	novel	-	-
36	R705R	m	57,3	current	IA	x	x	x	x	x	novel	-	-
36	L688F	m	57,3	current	IA	x	x	x	x	x	22885469	COSM306127	endometrium
37	P848P	f	74,4	never	IIIA	x	x	x	x	x	novel	-	-
37	V786L	f	74,4	never	IIIA	x	x	x	x	x	novel	-	-
37	A743T	f	74,4	never	IIIA	x	x	x	x	x	18258923	COSM26509	lung
38	L844L	f	52,5	current	IIA	x	x	x	x	x	novel	-	-
39	K867K	m	37,5	current	IB	x	x	x	x	x	novel	-	-
40	K745K	m	66,8	current	IIIA	x	x	x	x	x	novel	-	-
41	K713K	m	73,9	current	IIIA	x	x	x	x	x	novel	-	-
42	I780I	f	64,0	current	IIIA	x	x	x	x	x	novel	-	-
43	I780I	f	59,3	current	IA	x	x	x	x	x	novel	-	-
44	I780I	m	56,5	former	IIIA	x	x	x	x	x	novel	-	-
45	I706I	f	66,8	former	IB	x	x	x	x	x	novel	-	-
46	H773H	f	67,9	current	IV	x	x	x	x	x	novel	-	-
47	D829D	f	67,8	former	IV	x	x	x	x	x	novel	-	-
48	V851V	f	59,3	current	IB	x	x	x	x	x	23344264	-	endometrium
48	N842N	f	59,3	current	IB	x	x	x	x	x	novel	-	-
48	T710I	f	59,3	current	IB	x	x	x	x	x	novel	-	-
49	E804E	m	62,4	current	IIIA	x	x	x	x	x	-	COSM1451603	large intestine
50	A702A	f	71,4	former	IV	x	x	x	x	x	-	COSM1451570	large intestine
51	P848S	f	56,2	current	IIA	x	x	x	x	x	novel	-	-
52	V774S	f	49,6	current	IV	x	x	x	x	x	novel	-	-
53	R803Q	f	72,9	never	IIA	x	x	x	x	x	novel	-	-
54	Q791L	f	35,9	current	IIIA	x	x	x	x	x	novel	-	-
55	Q787 STOP	f	75,5	never	IIIA	x	x	x	x	x	novel	-	-

Patient ID	Sign of the aminoacid change	Gender	Age (diagnosis)	Smoking status	Stage at diagnosis	Drug	TKI line	Response	Censored	PFS	Reference PMID	COSMIC ID	Tissue distribution
56	N700T	f	71,4	former	IA	x	x	x	x	x	novel	-	-
57	M793V	m	76,3	former	IIB	x	x	x	x	x	novel	-	-
58	L788P	m	63,3	current	IIA	x	x	x	x	x	novel	-	-
59	R832C	m	76,1	current	IB	x	x	x	x	x	21057220	COSM28286	ovary
59	I789T	m	76,1	current	IB	x	x	x	x	x	novel	-	-
60	G708#	f	65,7	current	IIA	x	x	x	x	x	novel	-	-
61	G779G	m	60,5	current	IIA	x	x	x	x	x	novel	-	-
62	S811F	m	77,9	former	IV	x	x	x	x	x	22975805	COSM1187305	lung
63	V851I	m	48,1	current	IIA	x	x	x	x	x	23485129	COSM12727	lung
64	L862V	m	59,7	former	IIA	x	x	x	x	x	21841502	COSM133111	lung
65	E709A	m	62,5	former	IV	x	x	x	x	x	23344264	COSM13427	lung
66	E709A	m	68,6	current	IV	x	x	x	x	x	23344264	COSM13427	lung
67	G779S	m	57,6	former	IA	x	x	x	x	x	17626639	COSM25016	lung
68	I853I	f	61,5	current	IV	x	x	x	x	x	21057220	COSM53231	lung
68	D855G	f	61,5	current	IV	x	x	x	x	x	16870303	COSM28606	lung
69	E829K	m	70,6	current	IIIA	x	x	x	x	x	21070477	COSM53291	lung
70	T790A	m	58,3	current	IV	x	x	x	x	x	23546020	COSM1272071	esophagus
70	L692H	m	58,3	current	IV	x	x	x	x	x	-	COSM726863	lung
71	V843I	f	50,5	current	IV	x	x	x	x	x	21274259	COSM85894	lung
72	V834A	m	59,0	current	IB	x	x	x	x	x	20208477	COSM41665	prostate
73	Q820R	f	63,4	current	IIA	x	x	x	x	x	21266046	COSM85898	prostate
74	L704L	f	70,6	current	IIB	x	x	x	x	x	21057220	COSM53225	ovary
75	L798P	m	61,4	never	IIIA	x	x	x	x	x	-	COSM1451601	large intestine
76	L692F	m	71,1	former	IV	x	x	x	x	x	23912954	-	lung
77	V786A	m	N/A	never	IIIA	x	x	x	x	x	novel	-	-

x: Patient did not receive *EGFR* tyrosine kinase inhibitor therapy
E: Patient received erlotinib
G: Patient received gefitinib
* Patient carrying complex *EGFR* mutation including classic mutation
** Exon 20, 2284_2285 del1 frame shift
# Exon 18: 2122 A/C
N/A: Not available
Rows of synonymous mutations are highlighted in gray.

Supplemental Table 2. Correlation of clinicopathological features, outcome variables and *KRAS* mutational status in patients with advanced pulmonary adenocarcinoma (n=505)

		No. of patients (%)	**WT (%)**	**KRAS12 (%)**	**KRAS13 (%)**	***P value***
	All patients	**505 (100%)**	**338 (67%)**	**147 (29%)**	**20 (4%)**	
Response [c]	PD+SD	240 (47.5%)	157 (46.4%)	72 (49%)	11 (55%)	0.260
	CR+PR	245 (48.5%)	161 (47.6%)	75 (51%)	9 (45%)	
Survival	Median PFS (days) [d]		211 (189-32)	185 (156-214)	157 (0-323)	0.534
	Median OS (days) [d]		479 (395-63)	471 (329-613)	330 (185-475)	0.917

Data shown in parentheses are column percentages; ECOG PS, Eastern Cooperative Oncology Group performance status; PD, progressive disease; SD, stable disease; CR, complete response; PR, partial response, PFS, progression-free survival; OS, overall survival.

Supplemental Table 3. Major clinicopathological characteristics of the advanced-stage lung adenocarcinoma patient cohort with full clinical follow-up (n=419).

		Total	EGFR mutation			KRAS mutation	KRAS and EGFR wild-type	P value
			Classic EGFR mutation	Rare EGFR mutation	Synonymous EGFR mutation			
Total		419[#] (100%)	22* (5%)	26** (6%)	16* (4%)	80[##] (20%)	271[#] (65%)	
Age (mean±SD)		63.9±10.3	67.4±10.4	63.5±9.8	64.3±8.80	59.9±10.9	64.6±9.8	**0.003**
Gender	**Male**	203 (48%)	7 (32%)	13 (50%)	7 (44%)	37 (46%)	135 (50%)	0.571
	Female	216 (52%)	15 (68%)	13 (50%)	9 (56%)	43 (54%)	136 (50%)	
ECOG PS	**0**	185 (45%)	13 (59%)	13 (50%)	8 (50%)	35 (44%)	116 (45%)	0.709
	1-2	223 (55%)	9 (41%)	13 (50%)	8 (50%)	45 (56%)	144 (55%)	
	Unknown data	11	0	0	0	0	11	
Smoking status	**Never-smoker**	70 (17%)	9 (43%)	6 (23%)	3 (19%)	8 (10%)	44 (17%)	**0.028**
	Former smoker	134 (33%)	7 (33%)	6 (23%)	6 (37%)	34 (43%)	81 (32%)	
	Current smoker	198 (50%)	5 (24%)	14 (54%)	7 (44%)	37 (47%)	131 (51%)	
	Unknown data	17	1	0	0	1	15	
Tumor Stage	**Unresected IIIA**	68 (16%)	2 (9%)	5 (19%)	10 (63%)	16 (20%)	36 (13%)	**<0.001**
	IIIB-IV	351 (84%)	20 (91%)	21 (81%)	6 (37%)	64 (80%)	235 (87%)	

Data shown in parentheses are column percentages. Categorical parameters of the different mutational groups were analyzed by Chi-square test. Age as a continuous variable was analyzed in the mutational groups by ANOVA and Tukey's multiple comparison test.

[#] *KRAS* molecular analysis was not done in 4 cases.

[##] *EGFR* molecular analysis was not done in 54 cases.

* 2 concomitant *KRAS* mutation was identified.

** 2 concomitant *KRAS* mutation was identified and *KRAS* analysis was not done in 2 cases.

ECOG PS, Eastern Cooperative Oncology Group performance status.

Supplemental Table 4. Distribution of *EGFR* mutation status in TKI-treated patients.

		TKI therapy		
		Total	**1st line**	**2nd and 3rd line**
Total		151 (100%)	30 (20%)	121 (80%)
WT for KRAS and EGFR		98	0	98 (100%)
Synonymous EGFR mutation		9	2 (22%)	7 (78%)
EGFR mutation	**Total**	44	28 (64%)	16 (36%)
	Classic	24	17* (71%)	7* (29%)
	Rare	20	11** (55%)	9 (45%)

Data shown in parentheses are row percentages.
* In one patient concomitant *KRAS* mutation was identified.
** In two patients concomitant *KRAS* mutation was identified.