# MOLECULAR PATHOLOGY IN LUNG CANCER: A BRIEF REVIEW

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**ABSTRACT** Lung cancer is a heterogenous tumor which develops as a result of accumulation of multiple genetic, and epigenetic changes together with environmental pathogens. In the last decade, targetable specific molecular changes were described, and important developments in the treatment, and survival of these patients have been obtained. In recent years, with recommendations of international guidelines, analyses of molecular changes related to sensitivity to tyrosine kinase inhibitors as EGFR gene mutations , and ALK/ROS gene rearrangements have been performed in many laboratories in cases with advanced stage non-small cell carcinomas. Inclusion of BRAF, MET, RET, HER2, and KRAS genes in the next generation sequencing panels, and analysis of EGFR T790M mutations related to the resistance to tyrosine kinase inhibitors are recommended. As the knowledge, and studies about genes, and mechanisms increase, determination and analysis of the laboratory tests for the markers which play roles in the patient selection will develop.

**KEYWORDS** Lung cancer, gene, molecular pathology, analysis

## INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in men, and women all over the world. When compared with other frequently seen cancers as colon, breast, and prostate cancers, mortality rates differ significantly which can be typically explained by their detection at an advanced stage, and their being histologically, and biologically heterogenous tumors.[1-4]

Historically, lung cancers have been divided into two main categories as small cell, and non-small cell carcinomas based on their histological characteristics, and responses to conventional treatments.[5] Non-small cell carcinomas constitute nearly 80% of all lung cancers. Up to the last decade, all non-small cell lung cancers have been treated similarly just like homogenous conditions based on their clinical stages. In the last decade studies related to molecular basis of lung cancers, and their clinical reflections have demonstrated that different subtypes of lung cancers develop from disease-specific molecular pathways, and that some of these tumors display molecular characteristics which may be targeted therapeutically.[1-6] It is presently known that molecular status in non-small cell carcinomas has a critical importance at least comparable to histological phenotype, and a potential to effect clinical course of the disease, and its response to treatment.[7]

As a result of studies performed recently, specific molecular changes which direct tumor growth, and provide therapeutic targets have been best described for the subtypes of adenocarcinoma. Oncogenic driver mutations have been detected in more than 50% of lung adenocarcinomas, and their presence mostly excludes other driver mutations. As is the case with other malignancies tumorigenesis is related to activation of proteins regulating tumoral growth (KRAS, EGFR, BRAF, MEK-1, HER-2, MET, ALK, and RET etc.), and inactivation of tumor suppressor genes (p53, PTEN, LKB-1 etc).[8] Detectable driver mutation rate was found in 54% of 516 cases with stage 4 lung adenocarcinoma that were subjected to molecular test panel by Lung Cancer Mutation Committee, while amplification of KRAS (22%), EGFR (17%), ALK (7%), BRAF (2%), and MET genes(2%) were observed in respective percentages of cases. However, PIK3CA, HER2, MEK1, NRAS, and AKT1 were detected in less than 2% of the cases. These aberrations excluded each other in 97% of the cases. Other mutations namely TP53, NF1, STK11, KEAP1 were also characterized in these tumors.[9] In nearly 40%


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of squamous cell carcinomas, targetable molecular aberrations have been observed. Amplification rates of FGRF (20%), PTEN (10%), AKT1 (6%), DDR2 (4%), PIK3CA 84%) were detected as indicated. Besides TP53 (81%), BRAF, and EGFR mutations were observed.[10] In 3% of the tumors, MHC class 1 gene loss was seen which suggested potential role of immunotherapy in this molecular subtype.[11]

Technology, and knowledge supporting molecular tests rapidly accumulate, and in parallel with this increase targetable genomic alterations in lung cancer also increase in number. In this review we wanted to summarize clinically important, and most frequently modified information about oncogenic and tumor suppressive genes which are critically important both in the development of treatment strategies and also in increasing the level of understanding of molecular pathology of lung cancer, and to discuss methods recommended for the analysis of these genes in the light of guideline information.

## KRAS

KRAS belongs to RAS family of prootoncogenes, and encodes a G protein which has a critical role in the regulation of proliferation, differentiation, and survival of cells, and in the control of signal conduction pathways. Ras proteins are dependent on GDP, and they are silent in normal inactive cells. Activated GTP ligand plays a key role in the activation of growth hormone receptors. Activated Ras-GTP activates a series of pathways including RAS/RAF/MEK/MAPK pathway which contains mitogen activated protein kinase (MAPK) and PI3-K (PI3K/AKT/mammalian target of rapamycin-mTOR) pathway. Activating mutations changes GTPase activity of protein, prevents inactivation of active RAS-GTP into GDP, and leads to increase in signals along growth- promoting pathways. Activating mutation in KRAS oncogen is the most frequently seen (25-40%) oncogenic alteration in lung adenocarcinomas. When amino acids on codons 12,13 or 61 are displaced due to point mutations, Ras proteins gain oncogenic potential. In non-small cell lung carcinomas 97% of KRAS mutations are seen on codon 12 or 13.[12-13]

Frequency of KRAS mutations demonstrates differences among ethnic groups. Their incidence rates are 5-10% in Asians, 25-35% in white Europeans or Americans, and 15-25% in Africans.[1,4,8,13] History of smoking is detected in 90-95% of the cases with lung carcinoma. KRAS, and EGFR mutations are largely exclude each other, and they are rarely detected in the same tumor.[1] KRAS mutation is rarely seen in cases with squamous cell, and small cell carcinomas.[8]

Some studies have indicated that the presence of KRAS mutation is a negative prognostic factor in adenocarcinomas, while some others reported lack of any prognostic significance of KRAS mutation.[4-7] Rosell et al reported KRAS mutation as a negative prognostic marker for relapse, and morbidity in all stages, and histological types of the lung cancer.[14] In many studies on second-, and third- line treatment with EGFR tyrosine kinase inhibitors, correlation between KRAS mutation, and resistance to EGFR tyrosine kinase inhibitor treatment has been reported.[15-18] Many centers used KRAS mutation test as a component of the algorithm of molecular diagnosis , and a negative predictor in the evaluation of response to EGFR tyrosine kinase inhibitor treatment. However as data demonstrating worsened disease progression related to treatment with EGFR tyrosine kinase inhibitor instead of conventional platinum-based chemotherapy were accumulating, the decision to use EGFR tyrosine kinase inhibitors in the treatment of lung cancers was not made frequently as done previously unless results of EGFR test were obtained, thus diagnostic role of KRAS in lung cancers diminished. Presently, absence of KRAS mutation does not contribute clinically useful data to the result of EGFR mutation test, and it should not be used as a determinant of EGFR TKI treatment. However, since KRAS, and EGFR mutations are excluding each other, as a component of designed algorithm, a cost-effective, and rapidly performed KRAS analysis may be used to maximize test efficiency and to exclude KRAS mutation-positive tumors from confounding EGFR mutation tests.[1]

## EGFR

EGFR is a protein product of gene containing 28 exons spanning approximately 200 kb and it is localized on chromosome 7p11.2. EGFR has a region which binds N-terminal extracellular ligand, and a C-terminal intracellular region which comprise of transmembraneous lipophylic segment, and a tyrosine kinase domain. EGFR tyrosine kinase achieves proliferation, and survival of cells through autoactivation of EGFR or via PIK3CA/AKT1/MTOR and RAS/RAF1/MAP2K1/MAPK1 pathways.[19-21] Following binding to EGFR ligand, EGFR induces formation of homodimeric and heterodimeric receptors, and then intrinsic intracellular protein kinases activate. Dimerization process induced by binding to ligand results in cross autophosphorylation of key tyrosine residues. Signal cascade involving multiple number of pathways is activated leading to induction of multiple number of cellular responses as proliferation, differentiation, motility, and survival.[20-23]

Gain-of-function mutations or activating mutations of EGFR which induce tyrosine kinase activity were firstly determined in the year 2004 [1]. Incidence rates of EGFR mutations have been reported as 10-15% for Western, and 30-40% for Asian communities.[8,24,25] In non-small cell lung carcinomas, EGFR mutations are seen between the first exons of intracellular tyrosine kinase domains (exons 18, and 21). These mutations can be divided into 3 major categories as follows: frame deletions on exon 19; insertion mutations on exon 20, and missense mutations on exons 18-21. Most frequently (45%) frame deletions on exon 19 are seen, and they have more than 20 variants including delE746-A750. L858R is the second most frequently seen mutation localized on exon 21. These two mutations consisted of 85-90% of all EGFR mutations. Other EGFR mutations comprise of G719C, G719S, G719A, S720F on exon 18, and L861Q, L861R on exon 21. These mutations are associated with susceptibility to EGFR-TKIs. As has been demonstrated in many studies, with these treatment modalities more improved response, and progression-free survival rates, more easily tolerable, and superior quality of life have been achieved in the treatment of advanced stage non-small cell lung carcinomas when compared with platium-based chemotherapies. Exon 20 insertions which include point mutations on D770-N77insNPG, D770-N771insSVQ, D770-N771insG and T790M, V769L and N771T are frequently associated with resistance to EGFR-TKI treatment. T790M is the most important mutation on exon 20 which is detected in small number of patients with adenocarcinomas primarily resistance to EGFR-TKI treatment , and in most of the half of the patients secondary resistant to this treatment.[4-8,19,26,27] In patients having drug-sensitive EGFR mutation, resistance may develop 9-12 months after treatment . Second gain- of- function mutations in EGFR are most frequently related to mechanism of resistance, and exon 20 T790M mutation is detected in 50% of these mutations. These

patients are unresponsive to first-generation TKIs as gefitinib and erlotinib, and also second-generation TKIs as afatinib, and dacomitinib, while as a third-generation TKI osimertinib has been used as a treatment.[7,8,30]

EGFR mutation is frequently seen in Asians, youngsters, women, and nonsmokers, and in histological subtypes of adenocarcinomas. It is very rarely seen in pure squamous cell carcinomas, however EGFR variant III mutations, gain of copies, and protein overexpressions are more frequently seen in squamous cell carcinoma relative to adenocarcinoma.[8,19,28,29] EGFR mutation has been reported as the first molecular change in nonsmokers.[4,31]

## ALK

ALK encodes tyrosine kinase receptor which is normally expressed in some selected neuronal cell types, and found in a series of fusion proteins. Many balanced translocations which include ALK have been discovered. Among them the most frequently seen rearrangement involves EML4, and intracellular domain of ALK fuses with 'echinoderm microtubule associated protein-like 4 (EML-4). Breakpoints in ALK gene almost always occur on intron 19. EML4-ALK fusion most frequently occurs on chromosome 2p, as a result of short inversion [inv(2)(p21;p23)] which induces fusion between intron 13 of EML-4 and intron 19 of ALK.[3,4,6,718] Apart from EML4, more than 20 partners have been observed in less than 1% of ALK rearrangements including: KIF5B (kinesin family member 5b), TFG (TRK fused gene), and KLC-1 (kinesin light chain 1).[8,32] Oncogenic EML-4/ALK fusion protein has demonstrated kinase activating potential, and gain-of-function activities in both in vitro and in vivo models. ALK activation induces cellular proliferation, and inhibition of apoptosis, through RAS/RAF/MAPK1 PI3K/AKT ve JAK3-STAT3 signal pathways.[8,19]

In 3-7% of lung adenocarcinomas ALK rearrangements are seen.[1-8,19] Crizotinib which is a potent MET, ALK and ROS inhibitor, obtained FDA approval in 2013 , and provided effective treatment in metastatic non-small cell lung carcinomas with ALK rearrangements. With time, when cases that developed resistance to this treatment emerged, second-generation ALK inhibitors ceritinib, alectinib obtained FDA approval and started to be used in resistant cases.[33] Seventy-80% of ALK positive non-small cell lung carcinoma patients are nonsmoker young patients aged between 40, and 50 years. It is more frequently seen in Asians, and male patients with advanced stage disease at the time of diagnosis. Tumors of these patients are histologically non-squamous cell, and non-neuroendocrine subtypes. In some studies, it has been demonstrated that tumors with ALK rearrangements consist of more frequently mucin-producing solid or signet cell tumors in Asians, and tumors with acinar growth pattern in Western societies.[34,35] Although presence of ALK rearrangements has been considered as an excluding factor for driver mutations as EGFR, and KRAS mutations, rarely some studies have demonstrated their association with EGFR mutation.[9,37,38]

In some studies, frequent presence of lymph node metastases has been indicated in tumors which demonstrated ALK rearrangements relative to tumors with low T stage, and also shorter survival times when compared with EGFR mutant or wild patients.[6,39]

## ROS

ROS1 gene is localized on chromosome 6q22, and encodes a transmembrane tyrosine kinase receptor which demonstrates a high degree of homology with ALK in protein kinase domain.[8,33] Chromosomal rearrangements involving ROS1 gene, were firstly described in glioblastomas with ROS1-FIG gene fusion.[5,33] Apart from FIG, multiple number of fusion partners as KDELR2, TPM3, SDC4, LRIG3, and EZR have been defined. ROS 1 gene is observed in 1-2% of cases with non-small cell lung carcinoma.[1-8,19]. As is the case with ALK, ROS1 rearrangements are more frequently seen in young, nonsmoker patients, and among Asians.[8] The patients with ROS1 rearrangements, are susceptible to treatment with kinase inhibitors including ALK/MET inhibitor, crizotinib.[1-8]

## BRAF

BRAF gene encodes an effector downregulation protein of KRAS namely serine/threonine protein which is effective on the regulation of proliferation, and survival of cells , and it also activates MAPK signal conduction pathway.[4-8,19,38] Activating BRAF mutations are frequently observed in melanomas, while they are also seen in 1-3% cases of non-small cell lung carcinomas. Half of these mutations are activating V600E mutation localized on exon 15, other mutations include mutation G469A (39%) on exon 11, and D594G (11%) on exon 11 with indicated incidence rates. Apart from V600E, BRAF mutations are seen in smokers, while V600E mutation is seen in nonsmoker female patients. V600E mutations portend a worse prognosis relative to patients with wild type BRAF V600E mutation. Though BRAF inhibitors as vemurafenib, and dabrafenib have been reported to be effective in BRAF V600E positive advanced stage adenocarcinomas, clinical studies are still ongoing.[7,8]

## MET

MET oncogene is localized on chromosome 7q21-q31, and it encodes membrane tyrosine kinase receptor which is also known as hepatocyte growth factor.[4,7,8,19,39] Amplification of MET is observed in 1-7% of non-small cell lung carcinomas. MET amplification is gained thanks to a mechanism called 'kinase switch' and it causes acquired resistance to EGFR tyrosine kinase inhibitor.[7,8,19,39] Two major therapeutic strategies are being developed related to antibodies, and kinase inhibitors. Onartuzumab is a humanized monovalent antibody developed against MET, and in early phase clinical studies it has demonstrated promising synergistic effects with EGFR kinase inhibitors. Studies on MET kinase inhibitors mainly concerning crizotinib which is a potent MET kinase inhibitor are still ongoing.[7]

## RET

RET gene is localized on chromosome 10q11.2, and encodes receptor kinase which has a role in the development of neural crest. RET oncogene fusions (generally with KIF5B) are seen in 1-2% of the cases with lung adenocarcinoma. RET-KIF5B fusion has a transforming function, and occurs secondary to development of inversion between long, and short arms of chromosome 10. RET rearrangements are detected in nonsmokers, young patients, and in cases with poorly differentiated tumors. Multiple number of ongoing studies have found multi-kinase inhibitors to be effective on RET, and evidence of susceptibility to RET inhibition was observed in tumors which expressed KIF5B-RET fusion in in vitro setting. [7,8,40]

## HER2

HER2 gene encodes a membrane-dependent tyrosine kinase receptor belonging to ERBB family. HER2 activation is observed in only small proportion of lung cancers. Overexpression (20%), gene amplification (2%), and activated mutations (1.2%) related to HER2 gene are seen in respective percentages of the cases. Alterations in HER2 gene are frequently seen in adenocarcinomas, and mutations are observed in wild-type EGFR, and KRAS tumors. In some studies the presence of HER2 was associated with female gender, Asian ethnicity, and nonsmoker status[7,8] HER2 gene amplification is not consistent with HER2 mutation, and as is the case in breast cancer it has not any significance as a predictive, and prognostic marker. Results of preclinical studies have suggested the presence of an association between the presence of HER2 mutation, and resistance to the first-generation of TKIs [4,7,8]

## Molecular Test Guidelines

Molecular test guideline for lung cancer which also contains information about EGFR , and ALK molecular tests was published by College of American Pathologists (CAP), International Association for the Study of Lung (IASCL) and Association for Molecular Pathology (AMP), and it was revised in the year 2017.[1,2] In 2013 guideline, analysis of EGFR, and ALK was indicated as molecular tests which should be absolutely performed in lung cancer. In 2017 guideline ROS1 was also included in this group. The 2017 guideline indicates that in centers using next- generation sequencing in addition to 3 genes, if adequate material is obtained, then BRAF, MET, RET, ERBB2 (HER2) and KRAS may be included in an extended panel of tests.[2]

The 2017 guideline affirms recommendations of 2013 guideline concerning patient population that will be subjected to molecular tests . Molecular tests should be performed for advanced stage (stage IIIB, and IV) lung cancer patients at admission or untested low-stage lung cancer patients at baseline who demonstrate recurrences or disease progression. Clinical characteristics should not be used as exclusion criteria from molecular tests. Resection materials should be tested for the presence of mixed lung cancers which contain adenocarcinoma, and adenocarcinoma component. In biopsy, and cytology materials in which adenocarcinoma component can not be totally excluded, in cases with squamous cell, and small cell carcinomas clinical criteria (young age, and nonsmokers) may be used in patient selection for tests. In the determination of initial treatment, tests are equally appropriate both for primary, and metastatic tumor. Each one of metachronous primary lung carcinomas should be subjected to tests. Testing multiple different areas of a single tumor is not required. Test results should be reported within 10 working days.[1,2]

For EGFR mutation analysis, single gene tests (Sanger sequencing, pyrosequencing), real-time PCR, PCR based hotspot multiplex mutation tests (SNapShot Multiplex kit, MassARRAY SNP), and next-generation sequencing may be used.[1,2,8,19] The 2013 guideline suggests that the selected analytical method should be sensitive enough to be able to detect mutations in a sample which contains $\geq$ 50% malignant cells, while in 2017 guideline percentage of malignant cells dropped to 20%, so Sanger sequencing with low sensitivity is not recommended any more.[1,2] For PCR-based EGFR mutation analysis, specimens fixated with formalin, and embedded in paraffin, fresh tissue, use of frozen or alcohol fixated specimens are required, use of acidic or decalcification solutions, and heavy metal fixatives should be avoided.[2] In 2013 guideline use of cell blocks is preferred instead of blood smears , while 2017 guideline indicates that every sufficiently preserved cytology specimen with adequate cellularity may be used. Since 2/3 of the cases with lung cancer is at advanced stage at the time of diagnosis, in most of the patients only small amount of materials, and cytology specimens may be used. Pathologists should determine adequacy of tumor cell content, quality, and quantity of DNA of the specimen. In case of need, microdissection should be applied to enrich tumor cells. For patient selection for EGFR TKI treatment, methods as total EGFR immunohistochemistry or EGFR mutation specific immunohistochemistry, EGFR copy number analysis with FISH or CISH should not be used. In patients with lung adenocarcinoma carrying EGFR mutation, if progression occurs following EGFR-targeted TKI treatment, for 3. generation EGFR-targeted treatment, EGFR T790M mutation test should be used. The method to be used to detect this mutation should have the capacity to identify even 5% of mutant allele content.[1,2]

Many recent studies have demonstrated that lung cancer cells shed their DNAs (cfDNA) into circulation which can be detected with modern technologies as 'droplet digital' PCR, allele- specific PCR, NGS. In some cases, identification of plasma cfDNA from peripheral blood may be an alternative to detection of mutations from biopsy specimens.[41,42] Theoretical advantage of this method is that it provides derivation of tumor DNA in circulation ensourcing from multiple disease sites, and subsequently sampling of different tumor subclones with resultant detection of secondary clinical resistance. cfDNA analytical methods have higher analytical specificity, and very low false positivity rates (<5-20%), and demonstration of mutations under appropriate clinical setting may direct treatment with targeted inhibitors. However cfDNA analysis has a low sensitivity, and its negativity does not exclude possibility of mutation.[43,44] The 2017 guideline does not recommend use of plasma cfDNA molecular methods in the circulation for the primary diagnosis of lung adenocarcinomas. In cases where tissue biopsy material is inappropriate , inadequate or rebiopsy is not possible or tissue-based EGFR analysis can not be performed, as an alternative, molecular diagnostic method cfDNA may be used. The guideline recommends use of plasma cfDNA methods for the detection of EGFR T790M mutation in patients who demonstrate clinical resistance to EGFR-targeted TKI treatment or disease-progression.[2]

In 2013 guideline, use of 'dual-labelled break-apart' probes is advised for ALK FISH analysis with the aim to select patients eligible for ALK TKI treatment. Use of attentively validated ALK immunohistochemistry is recommended only as a screening method.[1]. With time data about ALK immunohistochemistry increased, two commercial clones (mouse monoclonal' 5A4 (Novocastra, Leica Biosystems, Buffalo Grove, Illinois), and rabbit monoclonal' D5F3 (Ventana) have demonstrated 95-100% sensitivity, and specificity when compared with ALK FISH. [45,46,47] Among these clones, D5F3 (Ventana) has received FDA approval for patient selection for ALK inhibitor treatment in the Unites States of America. In the 2017 guideline, immunohistochemistry is advised as an equivalent alternative of ALK FISH test. [2] In our country for reimbursements provided from social security institutes, ALK FISH test positivity is required.

The 2017 guideline recommends ROS1 gene analysis in all advanced stage lung adenocarcinomas independent from their clinical characteristics. As a method, FISH and RT-PCR are advised, besides use of ROS immunohistochemistry is recommended as

a screening test. This guideline also requires confirmation of positive immunohistochemical results using molecular or cytogenetic methods. [2]

## Conclusion

Prognosis of advanced stage lung cancer is poor, and expected average life span is only one year. In recent years, especially in patients with a subtype of adenocarcinoma who demonstrate some specific molecular changes, tyrosin kinase treatments directed to specific targets ensure marked improvements in survival rates, and quality of life. The importance of molecular tests increases every year, and continuosly new information, and applications come up to agenda. Understanding genes, and related mechanisms that are targeted by these molecules will allow better comprehension, and conduction of laboratory methods which are extremely important in the selection of specific patients having these changes.

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