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Current Concepts on the Molecular Pathology of Non-small Cell Lung Carcinoma

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Abstract

Recent advances in the understanding of the complex biology of non-small cell lung carcinoma (NSCLC), particularly activation of oncogenes by mutation, translocation and amplification, have provided new treatment targets for this disease, and allowed the identification of subsets of NSCLC tumors, mostly with adenocarcinoma histology, having unique molecular profiles that can predict response to targeted therapy. The identification of a specific genetic and molecular targetable abnormalities using tumor tissue and cytology specimens followed by the administration of a specific inhibitor to the target, are the basis of personalized lung cancer treatment. In this new paradigm, the role of a precise pathology diagnosis of lung cancer and the proper handling of tissue and cytology samples for molecular testing is becoming increasingly important. These changes have posed multiple new challenges for pathologists to adequately integrate routine histopathology analysis and molecular testing into the clinical pathology practice for tumor diagnosis and subsequent selection of the most appropriate therapy.

Keywords

adenocarcinoma; squamous cell carcinoma; targeted therapy; molecular testing; next-generation of sequencing

Introduction

Lung cancer is the leading cause of deaths in the United States and worldwide.[1] The high mortality associated with lung cancer is in part due to late diagnosis after regional or distant spread of the disease.[2] From biological and clinical perspectives, lung cancer is a heterogeneous disease with multiple histological subtypes, being the most frequent non-

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small cell lung carcinoma (NSCLC). Traditionally, NSCLC has been used to designate tumors that exhibit histological and cytological features different than small cell carcinoma (SCLC). Most NSCLCs can be grouped into three main categories: squamous cell carcinoma, adenocarcinoma and large cell carcinoma; however, there are other less frequently diagnosed histologic types.[3] Nowadays, due to the utilization of new therapeutic strategies and molecular diagnostic testing in NSCLC, particularly adenocarcinomas,[4] it is imperative that pathologists are more specific in the diagnosis of subtypes of NSCLC and they make sure that there is sufficient tissue or cytology sample for molecular testing.

In this review, we described the most frequently described targetable genetic abnormalities in NSCLC and discuss the current status and challenges of molecular testing in this disease, including the implementation of new molecular methodologies to better predict the outcome of the disease and select the appropriate therapy.

Clinically relevant molecular abnormalities of NSCLC

During the last decade, multiple molecular abnormalities affecting oncogenes and tumor suppressor genes have been described in NSCLC.[56] Of those, several gene mutations, amplifications and rearrangements have been identified as potential molecular targets. Here we review the characteristics of key cancer-related genes that have been emerged as potential targets in NSCLC using either tyrosine kinase inhibitors (TKIs) or monoclonal antibodies.

EGFR (epidermal growth factor receptor gene)

Mutations of *EGFR* in lung cancer are mostly limited to the first four exons of the tyrosine kinase domain (exons 18–21). The most frequent mutations are in-frame deletions in exon 19 (44% of all mutations) and missense mutations in exon 21 (41% of all mutations). These mutations are frequently diagnosed in lung adenocarcinomas (~20%–48%, vs. other NSCLC histologies ~2%), and strongly correlate with never-smoking status (50–60%), female gender (40–60%), and East Asian ethnicity (30–50%).[7] There are some reports suggesting that *EGFR* mutations are encountered most frequently in lung adenocarcinomas with non-mucinous differentiation and with a lepidic or papillary predominant pattern.[89] Activating *EGFR* mutations are biologically important because most of them have enhanced tyrosine kinase activity in response to epidermal growth factor stimulation.[210] *EGFR* mutations are diagnosed mostly using gene sequencing methodologies, although quantitative (q)PCR-based assays are also available. (Figure 1) There are some antibodies that identify mutant EGFR proteins, but they have not shown to be clinically useful.

The presence these *EGFR* mutations are clinically relevant because they have been associated with sensitivity to small molecule TKIs (gefitinib and erlotinib).[11–13] Unfortunately, some patients with activating *EGFR* mutations that respond initially EGFR TKIs subsequently relapse.[14] This resistance appears to occur through a range of different mechanisms, including most frequently, a second *EGFR* mutation (50%) in exon 20 (*T790M* and *D761Y*),[15] as well as other molecular mechanisms that include amplification of the *MET* oncogene (21%), [161718] mutations of *PI3KCA*, [192021 22] and epithelial-to mesenchymal transition (EMT) phenomenon.

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ALK (anaplastic lymphoma kinase gene)

In lung cancer, aberrant ALK expression has been identified in a subset of NSCLC, mostly adenocarcinomas. This abnormality consists in the formation of a fusion transcript with cell transforming activity and that is the product of a translocation of *EML4* (echinoderm microtubule associated protein like-4 gene) gene located at chromosome 2p21 and the ALK gene located at 2p23.[23] The encoded fusion protein with increased catalytic activity contains the N-terminal part of EML4 and the intracellular catalytic domain of ALK.[23] *EML4-ALK* rearrangements have multiple distinct isoforms with demonstrated transforming activity and that can be detected by multiplex reverse transcription-PCR methodologies.[2425] The EML4-ALK fusion positive tumors are detected in 2–7% of NSCLC,[2627] mostly adenocarcinomas arising usually in young never- or light-smokers patients.[28293031] Tumors with EML4-ALK translocation usually lack *EGFR* and *KRAS* mutations. [323334] ALK rearrangement has been mostly associated with an acinar pattern including a cribriform morphology and with signet ring cell features.[35]

It has been demonstrated that crizotinib, an oral inhibitor of the ALK and MET tyrosine kinases, showed that this drug is effective against advanced NSCLC carrying activated EML4-ALK translocation assessed by a fluorescence in situ hybridization (FISH) utilizing an ALK “break-apart” probe.[36] The cut-off criteria for positive ALK “break-apart” FISH test is the presence of >15% tumor cells having split ALK 5′ and 3′ probe signals, or had isolated 3′ signals.[30] (Figure 1) The overall partial and complete tumor response rate observed in patients with NSCLC tumors with positive FISH test and treated with crizotinib was shown to be of 57%, and the rate of stable disease was 33%.[26] It has been shown that patients with NSCLC *EML4-ALK* rearrangement treated with ALK inhibitors developed resistance.[37] The genetic alterations associated with documented acquired resistance to crizotinib are *ALK* amplification or secondary mutations within the kinase domain of gene (*L1196M*, *C1156Y* and *F1174L*).[38]

ROS1 (c-ros 1 gene)

This gene encodes for a tyrosine-kinase receptor of the insulin receptor family.[39] Gene rearrangements affecting *ROS1* with the development of oncogenic fusion protein have been identified in approximately 1% of NSCLC, and more frequently in younger, nonsmoking patients with adenocarcinoma.[4041] A phase I clinical trial has demonstrated that crizotinib has dramatic antitumor activity in patients with *ROS1*-rearranged NSCLC, with a high objective response rate of 57.1%.[42] Therefore, the identification of ROS1 fusion variants is important for personalized therapy in lung cancer, particularly in patients adenocarcinoma histology.[43444041] Up to now, a couple of fusion gene partners of *ROS1* have been identified in lung tumors.[4546]

RET

The tyrosine kinase receptor *RET* is involved in cell proliferation, migration and differentiation.[4748] *RET* mutations are known to incline to multiple endocrine neoplasia type 2 and sporadic medullary thyroid cancer.[48] A novel fusion oncogene between *RET* and *KIF5B* (kinesin family member 5B gene) was reported recently in lung cancer affecting approximately 1% of patients with lung adenocarcinoma, mostly young never smokers.[4950]

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KRAS (Kirsten rat sarcoma viral oncogene homolog gene) and *BRAF* (v-raf murine sarcoma viral oncogene homolog B gene)

KRAS is RAS family gene most frequently activated in lung cancer by point mutations detected in approximately 20% of lung adenocarcinomas, and more frequently found in patients with smoking history.[51] Most *KRAS* mutations are single amino acid substitutions in codon 12,13 and 61.[2,52] Ras signaling pathways are also activated in tumors in which growth-factor-receptor tyrosine kinases have been overexpressed.[53,54] Of great interest, *EGFR* and *KRAS* mutations in lung adenocarcinoma are mutually exclusive, suggesting different pathways to lung cancer in smokers and never smokers. KRAS mutations a have been associated to low response rates to EGFR-TKI therapies.[55] RAS is considered a not targetable molecule, therefore recent studies have evaluated the activation of the Ras downstream pathway, RAS/RAF/MEK, as a potential target for therapy in lung cancer.[56,57]

BRAF is a serine/threonine kinase that lies downstream of RAS in the RAS-RAF-MEK-ERK-MAP pathway.[58] The V600E *BRA*F mutation is frequently identified in melanomas.[58] *BRAF* mutations occur in 2 to 4% of with lung adenocarcinoma.[58,59,60,61] Most BRAF mutations detected in lung cancer are non-V600E mutations affecting exons 11 and 15, and they are mutually exclusive to *EGFR* and *KRAS* mutations.[62,63,64]

HER2 (human epidermal growth factor receptor 2 gene)

The incidence of *HER2* mutations ranges from 1 to 6 % of lung adenocarcinomas. In lung cancer, *HER2* kinase domain mutations (in-frame insertion in exon 20) and *EGFR* kinase domain mutations have similar associations with female gender, non-smoker and Asian ethnicity.[65,66,67,68] HER2 amplification has been reported in 4%–5% of NSCLCs and is also more frequent in the adenocarcinoma histology (8%).[69,70]

MET

This gene encodes for a receptor tyrosine kinase that activates multiple signaling pathways involved in cell proliferation, survival motility, and invasion.[71] *MET* amplification occurs in up to 7 % of NSCLC[72,16], and has been associated to resistance to targeted therapy in patients whose tumors harbor *EGFR* mutation treated with EGFR TKIs. [16,17,18]

PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha gene)

Phosphatidylinocitol 3-kinases (PI3Ks) are family of lipid kinase that play an important role in regulating cell growth, proliferation and survival.[27,37,4] *PIK3CA* mutations are found in approximately 1 to 3% of NSCLCs.[19,20,21,22] *PIK3CA* copy number gain (>3 copies per cell) is a common abnormality in NSCLC, predominantly in squamous cell carcinomas (33%–35 %) compared to adenocarcinomas (2%–6%).[21,75] PI3K, and its downstream effectors, *PTEN*, *mTOR* and *AKT*, are potential therapeutic targets for NSCLC therapy and are evaluated in clinical trials for lung cancer.[76] *PTEN* is a lipid phosphatase that inhibits PI3K-dependent signaling with tumor suppressor gene activity.[77] *PTEN* mutations are common in squamous cell carcinomas of the lung.[67,87,980] *PTEN* inactivation has been related decreasing EGFR TKIs sensitivity of *EGFR*-mutant lung tumors. [67]

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FGFR1 (fibroblast growth factor receptor type 1 gene)

This gene encodes a cell surface tyrosine kinase receptor of the FGFR tyrosine kinase family that includes four kinases (FGFR1 to 4). FGFRs play a critical role in cell proliferation and survival.[7481] It has been reported that *FGFR1* is somatically amplified in ~20% of squamous cell carcinomas and in 1–3% of adenocarcinomas of the lung.[828384] Currently, the preferred method to assess *FGFR1* copy number is FISH, but the definitions of copy number gain and gene amplification still need to be determined.

DDR2 (discoidin domain receptor 2 gene)

This receptor tyrosine kinase has been reported to be mutated in ~4% of squamous cell carcinoma of the lung.[8586] Mutations were found both in the kinase domain and in other regions of the protein sequence without hot-spots, which makes the analysis of mutations of this gene challenging. *DDR2*-mutant tumors have been suggested to respond to dasatinib therapy in patients with squamous cell carcinoma of the lung.

Molecular testing of lung cancer

The recent advances in NSCLC targeted therapy require the analysis of a panel of molecular abnormalities in tumor specimens, including gene mutations, amplifications and rearrangements, by applying different methodologies to tumor tissue specimens.[875] However, the diagnostic biopsy or cytology specimens available for molecular testing in advanced metastatic lung tumors are likely to be small specimens, including core needle biopsies (CNB) and/or fine needle aspiration (FNA), which may significantly limit molecular testing with currently available methodologies and technologies. It is known that both formalin fixation and paraffin embedding compromise the integrity of proteins and nuclei acids for molecular testing, particularly when non-buffered formalin is utilized and the specimens are fixed in formalin for greater than 24 hours. The cytology specimens are usually fixed in alcohol which is optimal for preservation of DNA. When the cytology specimen has abundant material, the sample can be fixed in formalin and processed as a cell block to obtain histology sections; both smears and cell block sections with abundant malignant cells can be successfully used for molecular testing in lung cancer. Few studies showed that the sensitivity of cell block specimens for molecular testing in lung cancer showing that, although slightly lower, are compatible to smears and ThinPreps.[88899091 92]

Currently, the surgical pathologist plays a crucial role on determining the appropriate therapy for patients with NSCLC. The handling of the biopsy and cytology specimens for pathological diagnosis and subsequent molecular testing requires thoughtful prioritization of the utilization of the sample to prevent the loss of tissue in less important analysis that the molecular testing requires for selection of therapy. (Figure 2) Also, the pathologist should determine if the amount of malignant cells available in the specimen is adequate for DNA extraction and also for histology section-based molecular tests (e.g., fluorescent in situ hybridization and immunohistochemistry).

On the other hand, our growing understanding of cancer biology of NSCLC, particularly the molecular evolution of tumors during local progression and metastasis, and the identification of molecular abnormalities developed after resistance to targeted therapies, emphasizes the

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importance of characterize the molecular abnormalities of the disease at every stage of its evolution. For molecular testing of advanced metastatic NSCLC is important to sample and analyze the tumors' specimen at each time point of clinical decision-making.[9376] In lung cancer, the National Comprehensive Cancer Network (NCCN), the American Society of Clinical Oncology (ASCO) and the International Association for the Study of Lung Cancer (IASLC)/College of American Pathologists (CAP)/Association of Molecular Pathology (AMP) recommended to use testing for *EGFR* mutations and *ALK* fusions to guide patient selection for appropriate TKI therapy in all patients with advanced stage adenocarcinoma, regardless of sex, race, smoking history, or other clinical risk factors.[949596]

New techniques for molecular testing

The most used technique for DNA mutation analysis is direct sequencing previous PCR amplification of extracted DNA. There are several of these methods available for mutation analysis of DNA extracted from FFPE tumor tissue specimens, including lung cancer. Sanger sequencing is one of the preferred sequencing methods to detect mutations of clinically relevant genes, such as the *EGFR* hot-spot mutations for selection of EGFR TKI therapy. It can detect essentially all base substitutions, small insertions and deletions. However, the main disadvantage is the relatively low sensitivity of mutant alleles, estimated to be ~20% of mutant vs. wild-type alleles,[97] and most importantly, its inability to examine multiple gene hot-spots simultaneously. The need for analysis of multiple genetic changes in small, clinically relevant biopsy and cytology specimens, has prompted to the development of multiplexed approaches for molecular testing, particularly for gene mutation analysis.

Multiplex genotyping methodologies

Multiplex PCR is defined as the simultaneous amplification of two or more DNA or cDNA targets in one reaction.[98] There are two major highly sensitive multiplex genotyping methodologies widely used for mutation analysis in lung cancer, the primer extension (SNaPshot®) assay (Life Technologies, Grans Island, NY) and the matrix-assisted laser desorption ionization time-off light (MALDI-TOF) mass spectrometry (Sequenom®, San Diego, CA). SNaPshot involves multiplexed PCRs, multiplexed single-base primer extension, and capillary electrophoresis. [99,100] Sequenom® involves multiplexed PCR and a mass spectrometry detection system. Both systems are able to detect multiple hot-spot mutation simultaneously using small amounts of DNA obtained from small FFPE biopsy specimens.[101102] These multiplex genotyping platforms are not designed for discovery purposes.

Next generation sequencing (NGS)

This technology has been available since 2004.[103] NGS has been applied to studies of DNA and RNA to examine the whole genome, exome, transcriptome and epigenome, and is rapidly changing the paradigm of lung cancer research and patient care.[103] Currently, commercially available NGS platforms include, among others, Illumina® HiSeq 2500 (Illumina Inc, San Diego CA), Personal Genome Machine (PGM™) and Ion Torrent™ systems (Life Technologies Grand Island, NY).[6] NGS technologies have been rapidly applied to clinical setting almost all tumor types. NGS can detect mutations, chromosomal

rearrangements and copy number alterations at high resolution.[5,104,76] Currently, clinical application of NGS is hampered by the large amount of data generated and the resultant and computational bioinformatics challenges needed for secondary verification, and the relatively high cost.[76]

Future directions

In lung cancer, molecular testing of tumors is usually performed using samples obtained for histological diagnostic intent and often using residual tissue specimens obtained from surgical resection procedures for treatment. However, based on our growing understanding of the molecular events associated to tumor progression and the mechanisms of resistance to targeted therapy, it is becoming clearer that molecular analysis should be applied directly to clinically relevant tumor specimens. This is important to consider in recurrences of surgically resected stages I–III tumors or refractory advanced metastatic chemotherapy-treated tumors obtained at treatment or diagnosis of the disease, respectively. These “old” samples may not reflect the current state of biomarkers after tumor progression or treatment with chemotherapy. Therefore, to ensure the most accurate assessment of a lung cancer patient’s disease and treatment responsiveness, their tumors should be molecularly characterized at multiple time points during the clinical decision-making process.

On the other hand, there are increasing concerns that intra-tumor heterogeneity of lung cancer can lead to underestimation of tumor genomics landscape portrayed from a single tumor biopsy and may present major challenges to personalized-treatment and biomarker development.[105,106] In lung adenocarcinoma, mixed populations of *EGFR*-mutant and wild-type cells have been reported and associated to reduced response to EGFR TKI.[107] Recently, it has been demonstrated in renal cell carcinoma that 73–75% of the driver genetic aberrations detected using NGS were sub-clonal, confounding the estimation of driver mutation prevalence. The presence of sub-clonal driver events in tumors, including lung, may provide an explanation for the inevitable acquisition of resistance to targeted therapeutics in advanced disease.[108,105]

Abbreviations

ALK	anaplastic lymphoma kinase gene
AMP	Association of Molecular Pathology
ASCO	American Society of Clinical Oncology
BRAF	v-raf murine sarcoma viral oncogene homolog B gene
CAP	College of American Pathologists
CNB	core needle biopsies
DDR2	discoidin domain receptor 2 gene
EGFR	epidermal growth factor receptor gene
FFPE	formalin-fixed and paraffin-embedded

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FGFR1	fibroblast growth factor receptor type 1gene
FNA	fine needle aspirations
HER2	human epidermal growth factor receptor 2 gene
IASLC	International Association for the Study of Lung Cancer
IHC	immunohistochemistry
KIF5B	kinesin family member 5B gene
KRAS	Kirsten rat sarcoma viral oncogene homolog
MALDI-TOF MS	matrix assisted laser desorption/ionization-time of flight mass spectrometry
NCCN	National Comprehensive Cancer Network
NGS	next-generation sequencing
NSCLC	non-small cell lung carcinoma
PCR	polymerase chain reaction
PI3K	phosphatidylinocitol 3-kinase gene
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha, gene
PTEN	phosphatase and tensin homolog gene
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ROS1	c-ros 1 gene
SCLC	small cell lung carcinoma
TKI	tyrosine kinase inhibitor
VIPR1	Vasoactive Intestinal Peptide Receptor 1 gene
WHO	World Health Organization

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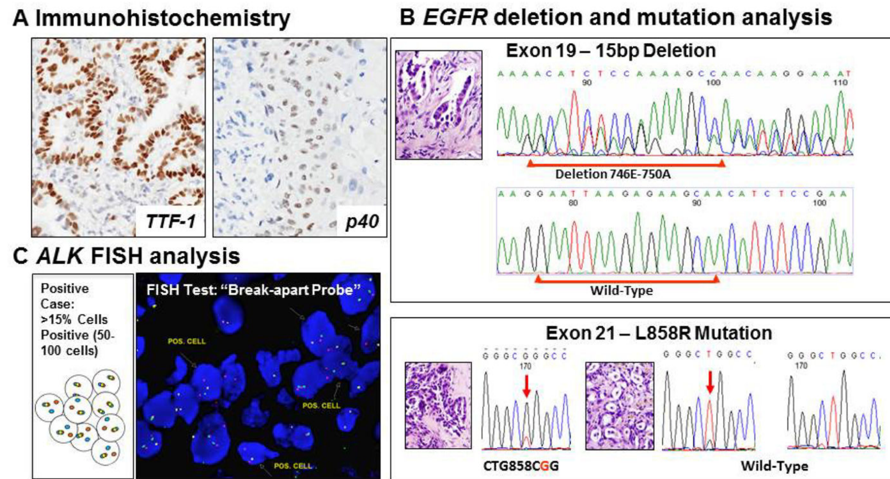

Figure 1. Histology section-based molecular tests for NSCLC

A. Immunohistochemistry panel: Thyroid transcription factor (TTF-1) is a marker of adenocarcinoma, and *p40* (*p63*) is a marler of squamous cell carcinoma. B. *EGFR* mutation analysis. C. *EML4-ALK* fusion fluorescent in situ hybridyzation (FISH) analysis.

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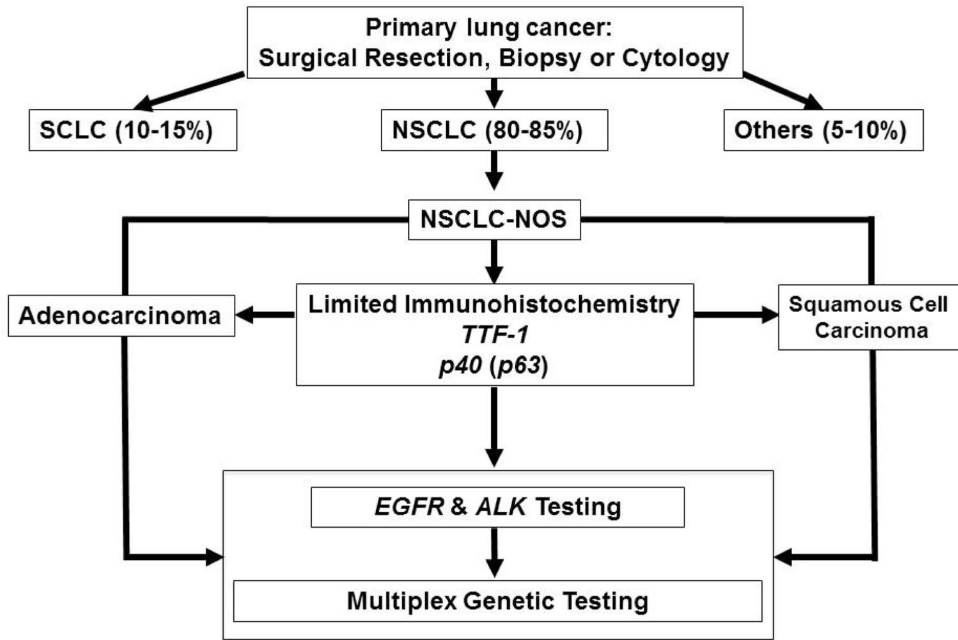

Figure 2. Histology subtyping based on treatment algorithm for lung cancer

The utilization of multiplex platforms to test mutations in tumor samples allows testing all NSCLC histologies for panel of mutations and other gene abnormalities regardless of their histology. SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; NOS, not otherwise specified