Review Article on New Developments in Lung Cancer Diagnosis and Pathological Patient Management Strategies

How should molecular findings be integrated in the classification for lung cancer?

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Abstract: The use of molecular diagnostics in the diagnosis and management of patients with advanced lung cancer has become widespread. Although molecular classification has increasingly been incorporated in the pathologic classification of certain types of human tumors (particularly within the hematologic, glial, and bone/soft tissue malignancies), genetic findings have not been formally incorporated into the pathologic classification of lung cancer, which presently relies solely on the assessment of histologic and immunophenotypic characteristics. Whether molecular classification should be adopted in lung cancer would depend on the diagnostic, prognostic, and predictive impacts of such classification—and whether these impacts confer significant values additive to those derived from the routine histologic and immunophenotypic assessment. We provide a brief overview on the genetics of lung cancer, including adenocarcinoma, squamous cell carcinoma, and neuroendocrine tumors (small cell carcinoma, large cell neuroendocrine carcinoma, and carcinoid tumors). We consider the values of molecular information with some examples, in terms of the current diagnostic, prognostic, and predictive impacts. Finally, we discuss the conceptual and technical challenges of adopting a molecular classification for lung cancer in clinical management for patients. While there are conceptual and technical hurdles to tackle in implementing molecular classification in the pathologic classification of lung cancer, such integrated histologic-molecular diagnosis may allow one to personalize and optimize therapy for patients with advanced lung cancer.

Keywords: Molecular diagnostics; molecular classification; lung cancer; EGFR; ALK

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Introduction

In the last few years, the use of molecular diagnostics has become widespread and integrated in the management of patients with advanced lung cancer. Nonetheless, genetic findings have not been formally incorporated into the pathologic classification of lung cancer, which presently relies solely on the assessment of its histologic and immunophenotypic features (1). Given the extensive published literature on both lung cancer and molecular diagnostics, each of which is a complex topic, we will not address these two topics in a comprehensive fashion. Instead, we focus on several salient aspects of lung cancer genetics in the framework of their impacts on lung cancer diagnosis and treatment.

Molecular classification has increasingly been incorporated in the pathologic classification of human tumors. In fact, the molecular status of certain genes is required for the pathologic diagnosis for some tumors (particularly within the hematologic, glial, and bone/soft tissue malignancies) in the World Health Organization

Table 1 Brief overview of molecular alterations of lung tumors in this review

Tumor type	Recurrent clinically/diagnostically-relevant genes	Select references
Adenocarcinoma	Oncogenic kinase drivers including *EGFR*, *KRAS*, *ALK*, *ROS1*, *RET*, *BRAF*, *ERBB2*, *NRG1*, *MET*, and genes in the NTRK family; tumor suppressors including *TP53*, *STK11*, *KEAP1*; *SMARCA4*	(5-23)
Squamous cell carcinoma	Tumor suppressors including *TP53*, PI3K pathway, and genes in the FGF receptor family	(24-27)
Adenosquamous carcinoma	(similar to those in adenocarcinoma)	(26-28)
Pleomorphic carcinoma	*MET*	(11-13)
Small cell carcinoma	Tumor suppressors including *TP53*, *RB1*	(29-31)
Large cell neuroendocrine carcinoma	*TP53*, *RB1*, *STK11*, *KEAP1*, *KRAS*	(32-34)
Carcinoid tumors	Epigenetic regulators including *MEN1*	(35-37)

(WHO) classification of tumors (2-4). Nonetheless, whether molecular classification should be adopted in lung cancer would depend on the diagnostic, prognostic, and predictive impacts of such classification—and whether these impacts confer significant values additive to those derived from the routine histologic and immunophenotypic assessment. We will discuss these considerations, as well as the conceptual and technical challenges of implementing a molecular classification in the clinical management of lung cancer.

Brief overview on our current understanding on the genetics of lung cancer

Histologically, lung tumors are classified into several categories: the most commonly encountered categories include adenocarcinoma and squamous cell carcinoma, rarely other non-small cell lung carcinomas (adenosquamous carcinoma, sarcomatoid carcinoma, and others). Neuroendocrine tumors of the lung comprise a distinct group that includes small cell carcinoma, large cell neuroendocrine carcinoma, and carcinoid tumors. Here, we will primarily focus on some of the more common tumor types listed above, rather than other rare categories (salivary gland-type tumors, papillomas, adenomas, and mesenchymal and lymphohistiocytic tumors) (1). As expected, given the morphologic diversity, lung cancer shows tremendous genetic diversity with multiple distinct and overlapping molecular subgroups, some of which are associated with histologic categories and subtypes (*Table 1*).

Genetically, lung adenocarcinoma comprises a collection of diverse subgroups; approximately 60–70% of tumors are known to harbor oncogenic driver mutations (such as in *KRAS*, *EGFR*, *BRAF*, or *ERBB2*), rearrangements (such as in *ALK*, *ROS1*, *RET*, *NRG1*, or one of the *NTRK* genes), or structural alterations (such as in *MET* with exon 14 skipping) (5,6). Some of these alterations appear to show histologic correlates. For instance *EGFR*-mutant lung adenocarcinomas often show an acinar or papillary-predominant pattern (so-called terminal respiratory unit phenotype) (7,8). *ALK*-rearranged lung adenocarcinomas typically show a solid-to-cribriform pattern with signet-ring cell features (9,10). Adenocarcinoma with *MET* exon 14 splice site mutation can be seen in up to ~30% of non-small cell lung carcinomas with pleomorphic histology (11-13). *NRG1*-rearranged lung adenocarcinomas often show invasive mucinous histology (14,15). However, these molecular-histologic correlates are not absolute, with diverse and overlapping histologic patterns reported in adenocarcinomas with those alterations mentioned above, as well as those with oncogenic rearrangements in *ROS1* (16,17), *RET* (18-20), or one of the *NTRK* genes (21). Identification of molecular subtypes in lung adenocarcinomas therefore relies primarily on molecular techniques, rather than histologic and immunophenotypic features alone (22,23). In the United States, according to the 2018 clinical testing guideline from the College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC), and Association for Molecular Pathology (AMP), the recommended "must-test" molecular markers for patients with advanced lung adenocarcinoma include *EGFR*, *ALK*, and *ROS1*, with an expanded panel that includes *KRAS*, *BRAF*, *MET*, *RET*, and *ERBB2* (22).

Lung squamous cell carcinoma is characterized by

frequent mutations in the PI3K growth factor signaling pathway and in tumor suppressor genes such as *TP53* (24,25). Unlike adenocarcinoma, squamous cell carcinoma typically lacks oncogenic driver mutations (such as *KRAS*, *EGFR*, and *BRAF*) or rearrangements (such as *ALK* and *ROS1*) as discussed above. Lung adenosquamous carcinoma, which contains both adenocarcinoma and squamous cell carcinoma with each comprising at least 10% of the tumor, has been shown to harbor mutations in *EGFR*, *KRAS*, and rarely *BRAF* (26-28) or *RET* rearrangement (20). Genetically, lung adenosquamous carcinoma thus resembles lung adenocarcinoma rather than lung squamous cell carcinoma.

Among primary neuroendocrine tumors of the lung, small cell carcinoma harbors inactivating mutations in both *TP53* and *RB1* in >90% of tumors (29-31), a subset showing *MYC* gene amplification (31). On the other hand, genetically, large cell neuroendocrine carcinoma is heterogeneous and comprises multiple subgroups that are small cell carcinoma-like, non-small cell carcinoma-like, and carcinoid-like (32,33). Mutations in *NTRK* family members have also been described in large cell neuroendocrine carcinoma but not in carcinoid tumors or small cell carcinoma (34). While carcinoid tumors are considered with small cell carcinoma and large cell neuroendocrine carcinoma within the group of pulmonary neuroendocrine tumors, carcinoids lack mutations in tumor suppressors and are genetically distinct from small cell carcinoma and large cell neuroendocrine carcinoma; instead, carcinoid tumors are characterized by a relatively low tumor mutational burden, with a subset of tumors harboring mutations in *MEN1* and epigenetic regulators such as *ARID1A* and *PSIP1* (35-37).

Diagnostic, prognostic, and predictive values of using molecular classification in lung cancer

Molecular diagnostics can be helpful in some instances in which the histologic and immunophenotypic features may be incomplete or inconclusive for the diagnosis. For instance, for a patient (particularly with light or never smoking history) diagnosed with squamous cell carcinoma by a biopsy, identification of mutations such as *EGFR* characteristic of lung adenocarcinoma would imply that the tumor is likely an adenosquamous carcinoma, with the adenocarcinoma component not been sampled (26,27). Another example involves a sarcomatoid tumor of the chest wall, in which distinction between pleomorphic/spindle cell carcinoma and sarcomatoid mesothelioma can be difficult (38). Identification of *MET* exon 14 splice site mutation (characteristic of a subset of pleomorphic/spindle cell carcinomas), if present, would strongly favor the diagnosis of pleomorphic/spindle cell carcinoma rather than mesothelioma. Furthermore, targeted next-generation sequencing can provide us the mutation status of dozens to hundreds of genes; with sufficient number of genes interrogated, mutational patterns can be derived (39,40) and potentially provide clues for the primary site in challenging cases. For instance, an ultraviolet mutational signature may suggest a tumor with a cutaneous origin (such as melanoma, squamous cell carcinoma); while a smoking-related mutational signature in a tumor from a smoker may suggest a primary lung malignancy (23) or other tumors that frequently harbor tobacco mutational signature such as head and neck squamous cell carcinoma (40).

Studies on the prognostic impacts of molecular subtype in lung cancer are ongoing, however with no definitive consensus currently. The association between *EGFR* mutation status and patient survival has both been reported and disputed in several studies including meta-analysis (41-43). Similarly, the published literature on the association between *ALK* rearrangement status and patient survival are not entirely consistent, with association reported in some studies but disputed in others (43,44). These inconsistencies may be due to the types of patient cohort and statistical methodology, since molecular characteristics (such as *EGFR* or *ALK* mutation status) can vary with clinicopathologic factors such as ethnicity, age, gender, smoking history (10,45-48), and histologic types (7-9,49), some of which correlate with survival and confound the association between molecular subtype and patient outcome. The effect on outcome also appears to vary depending on the particular *EGFR* or *ALK* alterations; for instance, among patients with *EGFR*-mutant lung adenocarcinomas, the best outcome was noted in those with *EGFR* exon 19 deletion mutations (50-52). Collectively, in a recent clinicogenomic study involving >28,000 non-small cell lung cancer patients (including >3,500 patients with advanced disease) in the United States, alterations in a National Comprehensive Cancer Network (NCCN)-listed driver gene (which includes *EGFR*, *ALK*, *ROS1*, *MET*, *BRAF*, *RET*, and *ERBB2*) are associated with longer survival in advanced disease (53). In a retrospective study on >2,100 non-small cell lung carcinoma patients with newly diagnosed brain metastases, the addition of *EGFR* and *ALK* mutation status information to previously known prognostic factors (such as age,

performance status, and number of cranial and extracranial metastases) improved the performance of the prognostic assessment (54), suggesting the values of incorporating molecular information in predicting outcome for this group of patients. Whether this can be generalized to patients in other settings or those with uncommon *EGFR* or *ALK* mutations remains unclear, however.

Molecular diagnostics has become incorporated in the treatment planning for many advanced lung cancer patients. For patients with advanced non-small cell lung carcinomas that harbor oncogenic sensitizing *EGFR* mutations, *EGFR*-targeted tyrosine kinase inhibitors have been shown to be superior to standard chemotherapy in both first-line and non-first-line settings (55,56). For patients with advanced non-small cell lung carcinomas that harbor *ALK* rearrangements, ALK-targeted inhibitors have emerged as the standard of care (57-60). Similarly, ROS1-targeted therapy has demonstrated efficacy in patients with advanced non-small cell lung carcinomas that harbor *ROS1* rearrangements (59). Combined BRAF and MEK inhibition has been used to treat patients with advanced *BRAF* V600E-mutant lung adenocarcinomas (61,62). Recently, TRK-targeted therapy has been shown to be efficacious across a wide range of *NTRK*-rearranged tumors regardless of the tumor origins including lung (63); identification of this molecular subtype in lung cancer, albeit rare [~0.2%; (21)], imparts therapeutic significance.

Conceptual challenges of using molecular classification in lung cancer

In addition to demonstrating values additive to histomorphology, molecular classification should be practical in its implementation. Molecular categories should be well-defined, each with consistent results derived irrespective of laboratories or techniques involved. While in theory simple, in practice there are several conceptual and technical challenges.

Since a classification system relies on the delineation of categories, what conceptually constitutes a molecular category? First, should each category be defined at the level of DNA (mutations, rearrangements, copy number alterations), RNA (expression status), or even epigenetic regulation? For instance, small cell lung carcinoma appears homogeneous genetically with frequent *RB1* and *TP53* mutations based on the mutation analysis (29-31) but transcriptionally comprises subgroups with distinct expression profiles, with one of the recent proposed nomenclature based on the expression of four factors: ASCL1, NeuroD1, POU2F3, and YAP1 (64). Currently, these four expression subtypes have no immediate prognostic or predictive impacts on the clinical management of small cell carcinoma. Although recent development to target ASCL1-positive small cell carcinomas using an anti-DLL3 antibody-drug conjugate have been discontinued due to toxicity and limited efficacy, clinical trials on other targeted therapies in small cell carcinoma are ongoing (65). Should small cell carcinoma be classified using one or several molecular categories then?

Second, how granular should each molecular category be defined? For example, would *EGFR*-mutant or *ALK*-rearranged lung cancer suffice—or should the exact mutations involved be specified? If latter, how many categories should there be, given the theoretically nearly infinite combinations of mutations in human tumors? While most *ALK*-rearranged or *EGFR*-mutant lung tumors respond to their respective targeted therapy, some of the alterations are resistant mutations rather than sensitizing mutations. Among *ALK*-rearranged lung adenocarcinomas, *ALK* fusion variants differ in outcome and resistance profile, with *EML4-ALK* variant 3 associated with increased incidence of secondary *ALK* resistance mutations including G1202R (66). Among *EGFR*-mutant lung adenocarcinomas, in addition to the activating and sensitizing mutations (most commonly *EGFR* L858R in exon 21 and in-frame deletion of the ELREA motif in exon 19), rare mutations that are resistant to most EGFR-targeted inhibitors (for instance *EGFR* exon 20 in-frame insertion) have been noted (67). Compound *EGFR* mutations, characterized by the presence of multiple mutations (some of which are uncommon) within a tumor, are also present in a subset of patients (68,69). Functional impacts of these mutations can be interrogated using high-throughput phenotyping (70); nonetheless, clinical significance of many uncommon *EGFR* variants remains poorly understood (71), posing substantial challenges on their classification.

Third, how should we handle the plasticity of the molecular category, especially given the increasing use of targeted therapy that provides selective pressure on the tumor? For instance, a subset of *EGFR*-mutant lung adenocarcinoma undergoes histologic transformation into small cell carcinoma as a mechanism of acquired resistance to EGFR-targeted therapy (72), with the risk of transformation increased by the presence of concurrent *TP53* and *RB1* mutations (72-74). Transdifferentiation of *EGFR*-mutant lung adenocarcinoma into squamous

cell carcinoma has also been reported as a mechanism of acquired resistance to EGFR-targeted therapy, including to first-line osimertinib (75); concurrent disappearance of *EGFR* T790M mutation in conjunction to squamous transformation has been reported in tumors resistant to second- or third-line EGFR-targeted therapy (76). Aside from *EGFR*-mutant tumors, histologic transformation of *ALK*-rearranged lung adenocarcinoma into small cell carcinoma (77,78) or squamous cell carcinoma (79) has been documented as a mechanism of acquired resistance to ALK-targeted therapy. In fact, histologic transformation is not unique to tumors treated by tyrosine kinase-targeted therapy and has been reported in *KRAS*-mutant lung adenocarcinomas resistant to immunotherapy (80). As targeted therapies evolve and become more widely adopted, transdifferentiation poses conceptual challenges on the molecular categories.

Fourth, can one tumor simultaneously belong to multiple molecular categories? While most driver mutations in lung adenocarcinoma (*EGFR*, *KRAS*, *ALK*, *ROS1*, and others) are generally considered to be mutually exclusive (81,82), rare tumors with concomitant driver mutations have been reported (83-85). These conceptual challenges outlined above would need to be addressed in the implementation of molecular classification in lung cancer.

Technical challenges of using molecular classification in lung cancer

Considering the technical challenges of molecular classification, one should be cognizant of the limitations from the molecular diagnostic tools, implementation logistics, and the tumor tissue involved.

What evidence does one need to classify a tumor within a molecular category? Currently, the most commonly utilized tools include single-gene sequencing assay, targeted next-generation sequencing (NGS), and fluorescence in situ hybridization (FISH). Immunohistochemistry for ALK and ROS1 has been used in the identification and confirmation of *ALK*-rearranged and *ROS1*-rearranged lung tumors, respectively (22). Also, multiplex transcript-based systems (such as NanoString) have been used for fusion detection in lung cancer (86,87). Nevertheless, given different performance characteristics and technical limitations inherent in each assay, each tool will invariably be associated with false-positive and false-negative results. For instance, neither break-apart FISH, DNA-based hybrid capture with NGS, and RNA-based anchored multiplex PCR with NGS (88) alone was entirely sensitive for detecting *ROS1* rearrangement in lung adenocarcinoma in one study (89); while another study reported that >20% of putative *NTRK* rearrangement in non-small cell lung carcinomas could not be confirmed by another molecular technique (21). Given that no single molecular detection tool is entirely sensitive and specific, a combination of tools using DNA-based NGS, RNA-based NGS, FISH, and/or immunohistochemistry (if available) may be needed to ascertain the molecular characteristics, particularly in tumors with unusual clinicopathologic characteristics.

In addition to suboptimal sensitivity/specificity, there are issues in implementation logistics in using molecular classification. Molecular testing is often neither cost-effective nor available in resource-limited settings. Since current reimbursement for molecular diagnostics is generally limited to patients with advanced disease, testing is rarely performed on early-staged tumors. One method to circumvent these limitations may be to use other surrogate tools to infer the molecular status. For instance, ALK immunohistochemistry has been optimized over the years and is currently considered to be equivalent to *ALK* FISH and NGS testing in identifying *ALK* rearrangements in lung cancer (22). Detection for most molecular alterations in lung cancer (such as *EGFR*, *KRAS*, *ERBB2*, *RET*, and *MET*) nonetheless relies primarily on molecular methods, with immunohistochemistry playing a minimal role currently (90).

Despite the importance of molecular status in therapy selection for many advanced lung cancer patients, the relevance of histologic analysis should again be emphasized. In addition to molecular testing, at least a portion of tumor tissue should always be preserved for histopathology; such practice can be challenging in cases with limited lesional tissue. Nonetheless, this allows one to ensure that there is sufficient lesional tissue for molecular testing, to exclude other etiologies for mass-forming lesions (such as infection), and to monitor for histologic transformation (such as small cell or squamous transformation) in patients treated by targeted therapy. Furthermore, lung is one of the most common sites for visceral metastases from diverse malignancies, and molecular testing results currently may not shed light on the tumor origin. While activating *EGFR* mutations are nearly exclusively found in non-small cell lung carcinomas, rearrangements of *ALK*, *ROS1*, *RET*, and *NTRK* have been reported in diverse tumor types (91). Mutations in *KRAS* or *GNAS* are present in a subset of carcinomas from diverse sites including lung, pancreas, and the gastrointestinal tracts, among others (92). Reliance

on molecular information alone devoid of clinical and histologic contexts can thus be misleading.

Conclusions

Large-scale genomic studies in the last two decades have transformed our understanding on lung cancer genetics. Molecular diagnostics has been integrated in many laboratories and general medical practice, transforming how we diagnose and manage patients with lung cancer. While there are conceptual and technical hurdles to tackle in implementing molecular classification in the pathologic classification of lung cancer, such integrated histologic-molecular diagnosis may allow one to personalize and optimize therapy for patients with advanced lung cancer.

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