



Lung Cancer

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Frequently Asked Questions

1. **What are the major types of lung cancers and how are they different in the molecular mechanisms of tumorigenesis?**
 - Lung cancer is a common cancer and the leading cause of cancer deaths in the Western world. Although the incidence and death rate have been declining since 1990 per statistics published by the American Cancer Society in 2020 [1], lung cancer is still ranked #2 of all new cancer cases (excluding basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder) in both males and females (estimated over 110,000 cases in each sex in 2020) and #1 in all cancer deaths (72,500 males and 63,220 females, both >20% of all cancer deaths) in the United States.
 - In clinical practice, the major lung cancer types are non-small-cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma, and other relatively less common types and small cell lung cancer (SCLC) [2]. Adenocarcinoma and squamous cell carcinoma comprise approximately 70%, SCLC 15–20% of all lung cancers [3].
 - Tobacco smoking has been recognized as the major cause of lung cancer, responsible for over 85% of all lung cancer cases [4]. The percentage of cigarette-smoking-caused lung cancers has declined thanks to the mass media campaigns and tobacco control policies [5, 6]. There are significant differences in the

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molecular tumorigenesis between cigarette-smoking-related and unrelated lung cancers. The tobacco smoke carcinogen-induced mutations are frequently G to T transversions, such as *RAS* codon 12 GGT (glycine) mutated to TGT (cysteine) or GTT (valine), and loss of function mutations of the tumor suppressor gene *TP53*.

- Overall, activating mutations of oncogenes are frequently seen in adenocarcinoma not related to cigarette smoking, resulting in self-sufficiency in growth signals, insensitivity to antigrowth signals, and evasion of apoptosis. The clinically significant alterations in this category include activating mutations in genes encoding the epidermal growth factor receptor (ERBB receptor tyrosine kinases) and related elements in the cell proliferation and survival signal pathways, and rearrangement involving *ALK1* or *ROS1*. Loss of function mutations of tumor suppressor genes are more common in tobacco-related SCLC and SCC. These mutations also occur in adenocarcinoma of nonsmokers but are usually not the driver mutations [3].
 - It is much more difficult to target a loss of function (tumor suppressor genes) than an activating alteration. Currently available targeted therapies, therefore, mostly apply to adenocarcinoma in non-smokers, whose tumors show so-called oncogene addiction. Small molecules inhibiting the protein kinases overactive due to genetic alterations represent the major breakthrough in the treatment of NSCLC. On the other hand, loss of tumor suppressor functions frequently results in accumulation of secondary mutations and higher mutation load in the tumor. These tumors are more likely to have neoantigens and, therefore, may respond better to immunotherapies, which is the second breakthrough of lung cancer treatment, clinically effective for the non-oncogene-addicted tumors.
- 2. What are the clinically significant genetic abnormalities seen in lung adenocarcinoma?**
- Multiple genetic abnormalities are associated with lung adenocarcinoma. According to the data archived at cBioportal.com, the most commonly mutated genes in lung adenocarcinoma are (in the order of highest to lowest) *TP53* (52.4%), *KRAS* (30.5%), *EGFR* (24.2%), *STK11* (16.8%), and *KEAP1* (15.8%); the genes most commonly involved in translocation/fusion are *ALK* (2.2%), *EML4* (2.0%), *ROS1* (1.5%), *CD74* (1.0%), and *RET* (1.0%). They can either be grouped into driver mutations versus non-driver mutations based on their roles in tumorigenesis, or into actionable mutations versus non-actionable mutations based on whether there are drugs targeting the specific mutation.
 - Currently, the actionable mutations/alterations in adenocarcinoma with approved drugs include [7, 8]:
 - ***EGFR* mutations:** Mutations occur throughout the *EGFR* (*ERBB1*) gene, resulting in abnormal activation of the EGFR kinase activity. Majority of the mutations occur in exon 18–21, encoding the protein kinase domain [9].
 - ***ALK* rearrangements:** *ELM4-ALK1* is the most common *ALK* fusion gene [10].
 - ***ROS1* rearrangements:** *ROS1* has various partner genes with *CD74* being the most common one [11].
 - ***MET* abnormalities** include exon 14 skipping and *MET* amplification, both leading to increased MET protein level and kinase activity [12].
 - ***BRAF* mutations:** *BRAF* mutations in lung adenocarcinoma have a different spectrum from that seen in melanoma; *BRAF* c.1799T > A; p.V600E only comprises approximately 50% of all *BRAF* mutations [13].
 - ***RET* rearrangements** are rare, present in only 1–2% of lung adenocarcinoma [14].
 - **Neurotrophic tyrosine receptor kinase (*NTRK*) fusions:** *NTRK1* fusion proteins are relatively common in lung adenocarcinoma; *NTRK2* and *NTRK3* fusions are present at low frequencies [15].
 - ***HER2*:** Mutations in the kinase domain of *ERBB2* (*HER2/neu*), one of the epidermal growth factor receptor or ErbB family tyrosine kinases, are found in approximately 1–4% of lung adenocarcinomas [16–18]. They are seen in tumors with a similar phenotype to those harboring *EGFR* mutations.
 - *EGFR* mutations are the most common targetable mutations in lung adenocarcinoma and have a high prevalence in younger, female, Asian non-smoker patients. The inframe deletion in exon 19 (see case 1 as an example) and c.2573T > G; p.Leu858Arg (L858R) mutation in exon 21 together comprise approximately 90% of all *EGFR* mutations, with the prevalence of exon 19 deletion slightly higher than that of L858R [9]. These two mutations are sensitive to the tyrosine kinase inhibitors (TKIs). The responsiveness to TKIs varies for the other less commonly seen mutations, such as *EGFR* c.2155G > A; p.G719S, c.2582T > A; p.L861Q, and c.2303G > T; p.S768I, etc.
 - In addition to the targetable *EGFR* mutations, there are also mutations associated with drug resistance. Identifying them helps to predict the responsiveness to targeted therapy. Two well-defined *EGFR* mutations are associated with resistance to first- and second-generation EGFR TKIs. Patients carrying these two mutations usually respond to therapy with a third generation TKI.
 - The *EGFR* c.2369C > T; p.Thr790Met (T790M) mutation is the most common mutation in *EGFR* that is responsible for acquired resistance to the first- and second-generation TKIs. The incidence of

T790M in treatment-naïve patients is less than 1%, whereas in first- and second-generation TKI-treated patients, it can be as high as ~50% [19, 20] (see case 2 as an example).

- *EGFR* exon 20 insertion is considered the major mechanism of refractoriness to the first- and second-generation TKIs in treatment-naïve patients [21].
- *ALK1* or *ROS1* rearrangement creates a fusion protein with constitutive tyrosine kinase activity. They occur in lung adenocarcinoma at low frequency, with *ALK1* rearrangements in 1–3% and *ROS1* rearrangements in 0.7–3.4% of Caucasian patients [22] [23]. In NSCLC, *ELM4* is the most common fusion partner of *ALK1*; other partner genes, such as *TGF*, *KLC1*, and *KIKF5B*, are rare [24]. The partner gene provides the dimerization domain required for the constitutive activation of *ALK1* kinase [24, 25]. More than 20 variants of *ELM4-ALK1* fusion proteins have been identified, all containing the intracellular kinase domain of *ALK1*, but variable length of *ELM4* due to different breakpoints. The trimerization domain (TD) of *ELM4* is required for the constitutive activity of *ELM4-ALK1* fusion protein. The presence and the length of the tandem atypical propeller (TAPE) domain of *ELM4* affect protein localization and sensitivity to *ALK1* inhibitors [10, 25, 26]. *CD74*, *SDC4*, *EZR*, and *SLC34A2* are well-documented *ROS1* fusion partners, among which *CD74* is the most common one, comprising approximately 38% of all *ROS1* fusions [11]. *ROS1* can also be rearranged to partner with *TPM3*, *MYH9*, *CCDC6*, *FIG*, *LRIG3*, *KDELRL2*, *MSN*, *TMEM106B*, *TPD52L1*, *CLTC*, and *LIMA1* [27]. Unlike *ALK1* and *ROS1* rearrangements that have multiple breakpoints, *ROS1* fusions with *CD74* and *EZR* often occur at intron 33, whereas breakpoints at intron 31 are more frequently seen in fusions with *SDC4* and *SLC34A2* [11]. The underlying mechanisms of how the fusions lead to *ROS1* kinase activation is currently unknown. The presence of *ALK1* or *ROS1* rearrangement predicts sensitivity to *ALK1* or *ROS1* kinase inhibitors. Due to a high degree of homology between the kinase domains of *ALK1* and *ROS1* [28], most of the currently available *ALK* inhibitors including crizotinib, ceritinib, lorlatinib, etc., also show cross inhibitory effect on *ROS1* fusion proteins [29].
- *MET* exon 14 skipping refers to the loss of exon 14 due to mutations at the splice site or nearby introns. Exon 14 skipping and mutation of codon Y1003 prevent Casitas B-lineage lymphoma proto-oncogene (c-CBL) E3 ligase binding, resulting in decreased protein degradation and increased *MET* protein level, enhanced *MET* phosphorylation, and prolonged *MET* activation [12]. *MET* amplification is frequently seen in acquired

resistance to *EGFR* inhibitors but also occurs in treatment-naïve patients. The increased *MET* activity due to exon 14 skipping and *MET* amplification are both sensitive to *MET* inhibitors [12, 30].

- *RET* rearrangement is rare in NSCLC with a frequency of 1–2% [14]. The *RET* fusion genes consist of the *RET* kinase domain and a dimerization domain provided by its partner gene. The dimerization confers constitutive *RET* kinase activity of the fusion protein [31]. Patients carrying *RET* fusion genes respond to multi-target tyrosine kinase inhibition; however, their clinical outcome is inferior to those with other oncogene-addicted NSCLC receiving selective TKIs [32].
 - *BRAF* mutations in lung adenocarcinoma include c.1799T > A; p.Val600Glu (V600E), and non-V600E mutations, with an approximate ratio of 1:1. V600E mutation occurs in 1.5–3.5% of lung adenocarcinoma; non-V600E mutations can either be activating or inactivating [13]. *BRAF* rearrangements in lung adenocarcinoma are rare [33]. A novel fusion *SND1-BRAF* has been reported in lung adenocarcinoma in non-smokers with increased phosphorylation levels of *MEK/ERK* and cell proliferation by in vitro study [34].
 - *NTRK* fusions in lung adenocarcinoma involve all three isoforms of *NTRKs*. *NTRK1* rearrangements are present in approximately 3%; *NTRK2* and *NTRK3* rearrangements in less than 1% of lung adenocarcinomas [15, 35]. There is a great diversity of *NTRK* gene partners, which render constitutive kinase activity to the fusion proteins and sensitivity to TKIs [36]. *MPRIP-NTRK1*, *CD74-NTRK1*, and *TMP53-NTRK2* are the relatively frequent fusions in lung adenocarcinoma [37].
 - *HER2* mutations occur in exon 20 (insertion), the kinase, transmembrane, and extracellular domains; some of them may have concurrent *HER2* amplification [18]. *HER2*-mutated lung cancers are sensitive to kinase inhibitors like dacomitinib [38] and afatinib [17], *HER2* monoclonal antibody trastuzumab [16] and ado-trastuzumab emtansine, a *HER2*-targeted antibody-drug conjugate [18].
3. **What is the mutation landscape of squamous cell carcinoma of the lung?**
- Squamous cell carcinoma (SCC) has a strong association with tobacco smoking. Despite the relatively higher mutation burden, the typical tyrosine kinase gene mutations/aberrations seen in adenocarcinoma are rare in squamous cell carcinoma [39–41]. Mutations/aberrations detected in squamous cell lung carcinoma are similar to the squamous cell carcinoma from other organs, referred to as “squamousness” gene signatures [42, 43].
 - The most common genetic alterations in lung SCC occur in the cell cycle regulator genes, including

deletion and loss of function mutations in *TP53* (>75%) and *CDKN2A/B* (~70%) [39, 41]. *TP53* mutation-associated signature is a significant prognostic biomarker and potentially predictive of response to immunotherapies [44].

- Receptor tyrosine kinase (RTK) gene amplifications are present in >30% of lung SCC, with *EGFR* and *FGFR1* being most common; however, mutations/fusions are rare [40, 45]. *FGFR1* amplification is associated with better prognosis [46]. *ERBB* family mutations are seen in >20% of lung SCC; however, clinical responses to TKIs were not promising in the patients harboring these mutations, suggesting they are unlikely driver mutations [43].
 - RAS, MAPK, and PI3K signaling pathway dysregulation is another feature of SCC, most commonly loss of *NF1*, amplification or mutation of *PIK3CA*, and loss of *PTEN*. The potential value for targeted therapy of these alterations is under investigation [40].
 - Squamous cell carcinoma has been shown to have a higher tumor mutation burden (TMB) than adenocarcinoma [47]. In recent years, TMB has evolved as a novel biomarker to predict the sensitivity to immune-checkpoint inhibitors (see answers to question 15).
- 4. When is mutation testing for squamous cell carcinoma indicated?**
- Given that there is no effective targeted therapy for lung SCC in routine clinical practice, mutation profiling is not recommended for well-defined squamous cell carcinoma by the current practice guidelines [48].
 - Approximately 0.4–4% of all lung carcinomas contain both adeno and squamous components under the light microscope, and the frequency is even higher based on the ultrastructural appearance [49, 50]. These tumors may be sensitive to targeted therapies if related mutations are present [48]. Therefore, if the specimens from lung cancer, such as a small biopsy or needle aspiration, are not representative of the whole tumor, or the morphologic and immunophenotypic features are not entirely characteristic of SCC and a definitive classification is challenging, pathologists should consider mutation testing of the sample (see example case 4).
 - The current expert consensus opinion is that oncologists or pathologists may consider molecular testing in tumors other than adenocarcinoma when the probability of a targetable oncogenic driver is high based on the clinical features of the patient, including young age (<50 years) and absence of tobacco exposure history [28, 48, 51].
 - Although routine clinical testing of TMB has not been standardized, TMB assessment together with mutation profiling with targeted panel next generation sequenc-

ing (NGS) is a hot topic for molecular testing of NSCLC, including SCC [52–54].

5. What is the mutation landscape of small cell lung cancer?

- Small cell carcinoma of the lung (SCLC) is a high-grade neuroendocrine tumor, possessing the worst prognosis among all lung carcinomas. There has not been any targeted therapy for SCLC so far. Recent genomic studies of SCLC reveal a complex genomic profile [55].
 - Both copy number gains and losses involving multiple chromosomes have been identified using array-based genomic analysis. Recurrent loss of 3p (*FHIT* and *RASSF1*), 17p (*TP53*), 13q (*RBI*), and 10q (*PTEN*) have been reported in multiple studies. The other commonly seen copy number gains/amplifications include 1p, 2p, and 8q harboring the *MYC* family genes [56–63].
 - SCLC has a high mutation rate partly attributed to exposure to tobacco carcinogens. Several pathways are involved in the mutations associated with SCLC. Deletion and/or loss of function mutations in *RBI* and *TP53* occur in >90% of the SCLC [63].
 - Alteration of telomere length is frequently detected in neuroendocrine carcinomas of the lung, greater in high grade forms (large cell neuroendocrine carcinoma and SCLC) [64]. *TERT* copy gain, together with *RBI* deletion, was an independent predictor of poor prognosis in a study by Simbolo et al. [65].
 - Large cell neuroendocrine carcinoma (LCNEC) is another form of high-grade carcinoma of neuroendocrine origin. There are similarities in the gene expression profiles suggesting a common origin of LCNEC and SCLC [66], and their molecular profiles are significantly different from that of low grade neuroendocrine tumors [67]. Molecular genetic distinction between LCNEC and SCLC has also been reported [68, 69]. However, the inconsistency of these findings and the clinical significance of the differences warrant further study.
- 6. Are there any clinically significant genetic alterations associated with other relatively uncommon lung cancer types?**
- Some of the other relatively uncommon lung cancer types included in the fourth edition of WHO Classification [2] are carcinosarcoma, pulmonary blastoma, NUT carcinoma, primary pulmonary lymphoepithelioma-like carcinoma (PLELC), and salivary-gland-type lung carcinomas.
 - Carcinosarcoma is an aggressive NSCLC that frequently contains a variety of components, with phenotypes ranging from adenocarcinoma, SCC, to different sarcomas. The mutation profiles corresponding to

different components may be present in one tumor. Multiple studies have reported that *TP53* mutations are frequently present, followed by *KRAS* mutation. Targetable mutations associated with adenocarcinoma, such as *EGFR* mutations and *ALK* rearrangement, are rarely detected, but *ALK* and *EGFR* amplification and overexpression are not uncommon [70–77]. One study found a high frequency of actionable *MET* mutations (8 of 36 cases tested) [78].

- Pulmonary blastomas are frequently associated with missense mutations of *CTNNB1* [79, 80], resulting in aberrant nuclear/cytoplasmic localization of beta-catenin protein and activation of the Wnt signaling pathway [81].
- No clinically significant mutation profile has been discovered in PLELC so far. One study found low TMB in PLELC despite relatively high frequency of *TP53* mutations [82].
- Salivary-gland-type lung carcinomas arise from the submucosal glands of the airway, accounting for <1% of all lung tumors [83]. Histologically, they resemble their salivary gland counterpart, and most of them also share the same genetic alterations. Table 7.1 lists the major types of pulmonary salivary gland tumors and their associated genetic signatures.
- NUT carcinomas carry the characteristic *NUT* rearrangement. The fusion partners are most commonly (70%) *BRD4* resulting from t(15;19) [84, 85]. *BRD3*, *BRD4*, and *NSD3* are other common gene partners of *NUT* [86]. The fusion protein causes epigenetic deregulation, resulting in loss of cell differentiation [87]. Detection of the *NUT* rearrangement in challenging cases provides evidence for a definitive diagnosis.

7. What is the purpose of molecular genetic testing for lung cancer?

- As a standard of care, the purpose of performing mutation profiling for lung cancer in routine clinical practice is to select patients for mutation-based targeted therapy. In patients who developed resistance to a targeted therapy, mutation profiling is an important approach to search for resistance mechanisms.
- When multiple lesions are present, mutation profiling may provide useful information to evaluate the origin

of the different lesions, the clonal diversity, and clonal evolution (see questions 10 and 11 below).

- Detecting characteristic genetic alterations provide definitive or additional supporting evidence for the diagnosis and classification of some special types of carcinomas (see answers to question 6 above).
- Beyond the standard of care recommended by the practice guidelines, molecular tests should also be considered when there is compelling evidence from clinical trial results that investigational targeted therapies are effective. TMB is considered a promising biomarker independent of PD-L1 immunostaining to select lung cancer patients for immunotherapies.

8. What genes/mutations should be tested for non-small-cell lung cancer?

- Genes with targetable mutations associated with non-squamous NSCLC are recommended by several published guidelines to be tested as the current standard of care [8, 48, 88]:
 - *EGFR* mutations, and *ALK1* and *ROS1* rearrangements must be tested as a separate single gene or panel test, for all lung adenocarcinoma patients. *BRAF*, *MET*, *RET*, *KRAS*, and *HER2* testing should be included in a larger panel whenever appropriate, if there is sufficient sample available [48].
 - *KRAS* mutation single gene test can be performed separately because of the high prevalence and the mutual exclusivity of *KRAS* mutation with *EGFR* mutations and *ALK* fusions in NSCLC. If *KRAS* mutation is detected, larger panel testing for other mutations is not necessary. Currently, there is no compelling evidence to support single gene tests for *BRAF*, *MET*, *RET*, and *HER2* [48].
 - The first-generation *NTRK* inhibitors, larotrectinib and entrectinib, were approved by the US Food and Drug Administration (US FDA) in 2017 for the treatment of any *NTRK*-fusion-positive cancers, including NSCLC [89]. When clinically indicated, testing for *NTRK* fusions in the absence of other driver alterations is recommended in the newer guidelines [8].
 - TMB testing to help selecting patients for immunotherapy [90], although not included in the guideline published in 2018 [48], may be considered for some patients with metastatic NSCLC [91].
 - Given the high prevalence of *EGFR* T790M mutation in acquired resistance to first- and second-generation EGFR TKIs, *EGFR* T790M must be tested in the setting of disease progression while on targeted therapy to select patients for third-generation TKIs [48].
 - Although mutations in the *ALK1* gene may be associated with drug resistance, current evidence is not sufficient to support routine testing for *ALK1* mutations.

Table 7.1 Pulmonary salivary-gland-type carcinomas and their genetic signatures

Tumor type	Genetic alteration
Mucoepidermoid carcinoma	<i>MECT1-MAML2</i> fusion
Adenoid cystic carcinoma	<i>MYB-NFIB</i> fusion
Pleomorphic adenoma and carcinoma ex pleomorphic adenoma	<i>PLAG1</i> rearrangement, <i>HMGA</i> amplification
Myoepithelial carcinoma	<i>EWSR1</i> rearrangement
Hyalinizing clear cell carcinoma	<i>EWSR1-ATF1</i> fusion

- *TP53* is altered in >40% of NSCLC and >80% of lung SCC [92]. Most clinical studies suggest that NSCLC with *TP53* alterations carry a worse prognosis and may be relatively more resistant to chemo- and radiation therapies [93]. Recently, *TP53* co-mutations in *EGFR*-mutated NSCLC have also been associated with poor outcome of TKI therapy [94]. *TP53* is usually included in the NGS panel for lung cancer mutation profiling to provide prognostic and predictive information.
 - There are many genes/mutations associated with NSCLC for which targeted or specific therapies are in development. Testing these genes/mutations may be considered in specific patient groups to provide information for clinical trial recruitment.
9. **When should a lung cancer sample be tested for mutation profile?**
- Lung cancer mutation testing/profiling is only required in patients presenting with advanced-stage (stage IV) or metastatic disease who are suitable for therapy, either at initial diagnosis or recurrent disease with progression, if not previously tested [8, 48, 88].
 - Molecular testing is encouraged for early-stage lung cancer [48]. Testing lung cancer samples from early-stage patients allows for obtaining molecular profiling results from high-quality resection specimen when it is readily available. These results are saved for the future in case the lung cancer progresses and it becomes necessary to have mutation profile results but it is difficult or unnecessary to obtain more sample. However, with the improvement of clinical management, a subset of early stage patients will be cured by surgical resection and/or chemoradiation therapy, thus, never needing the mutation profile. Each institution should set its own policy on testing patients with early-stage diseases.
 - Repeat testing on recurrent tumor or a blood sample (for circulating cell free tumor DNA) can be considered for patients who developed resistant or refractory disease to investigate the molecular basis of resistance and search for potential mutation targets to enroll the patients in clinical trials.
 - When a diagnosis is established and it is determined that molecular testing is necessary, tissue samples should be prioritized for molecular testing before exhaustion for other studies, especially when only cytology or limited biopsy samples are available. When the specimen size is small and additional studies are anticipated, it could be very helpful to cut multiple additional unstained sections upfront to avoid later refacing the tissue block for additional sections, which could result in significant loss of the limited tissue.
10. **When multiple lung cancer lesions and/or multiple metastatic tumors are identified and excised/biopsied in one patient, should each of them be tested for mutation profile separately?**
- There is no significant clonal diversity in a tumor that would cause sampling bias in the molecular testing of NSCLC [95, 96]. Furthermore, the mutation patterns remain quite stable in metastatic and recurrent tumors [95]. Therefore, primary or metastatic tumors are equally suitable for molecular testing [97]. It is not necessary to test different regions of one tumor for mutation profiles, and routine testing of metastatic or recurrent lesions of a known primary NSCLC is not recommended.
 - In patients with synchronous multifocal lung cancers, these lesions can be metastatic from one primary or concurrent multiple primary lung cancers [98, 99]. Distinguishing multiple synchronous primary from metastatic malignant masses is important for lung cancer staging and has significant treatment implications because the treatment protocols for metastatic late-stage lung cancer is substantially different from early-stage disease (see case 3 as an example).
 - Comparing the histomorphologic features of multiple tumors can be a powerful tool to determine whether they are metastatic late-stage cancer or multiple primaries. However, histologic features are not always reliable [100]. Furthermore, when a metastatic cancer is identified in a patient with a history of more than one cancer of the same histologic type (i.e., adenocarcinoma of different organ origin), it could be more challenging to determine the origin of the metastatic cancer based on histologic and even immunophenotypic features.
 - Molecular profile as definitive clonal evidence for a tumor sample has been used to study clonal diversity, clonal evolution, and intra-tumor heterogeneity [95–97, 101]. Combining histomorphologic and genotypic assessments is the ultimate approach to the accurate staging of lung cancer and defining the origin and evolution of a metastatic cancer [102, 103].
 - The decision of whether to test each of the tumors should be based on the clinical context of the patients, and the communications between pathologists and clinicians [88]. Recurrent tumors are recommended to be re-tested only when there are clinical indications, such as progression under targeted therapy suggesting acquired resistance, pathologic evidences suggesting clonal evolution, or a different tissue/organ origin.
11. **How are different types of genetic aberrations of lung cancer tested in the clinical diagnostic laboratories?**
- Many different molecular methods can be used to detect mutations and/or other genetic alterations in

NSCLC. Because only a few genes/alterations (*EGFR* mutations, *ALK1* and *ROS1* translocations) are mandatory to be tested, single-gene or small panel tests are still widely performed in clinical diagnostic laboratories. After the first practice guideline published in 2013, some manufacturers have made commercial test kits focused on NSCLC mutations. The characteristics, advantages, and disadvantages of different methods used in molecular tests of NSCLC are summarized in Table 7.2.

- Point mutations, either as single- or multi-nucleotide variants, frequently occur in *EGFR*, *KRAS*, *BRAF*, *HER2*, and many other genes and are detectable by almost any molecular testing methods, if designed appropriately. Mutant-allele-specific polymerase chain reaction (PCR) needs special primer design to detect multinucleotide variants (MNVs); the commercially available allele-specific primers or probes are usually not designed for the multinucleotide changes. For example, allele-specific PCR designed for *BRAF* c.1799T > A; p.V600E would not accurately detect less commonly seen c.1798_1799delinsAA; p.V600K or c.1798_1799delinsAG; p.V600R mutations. Droplet digital PCR method is a variant form of PCR that markedly increases the analytic sensitivity and quantitation accuracy. It is particularly useful when the sample contains a low percentage of tumor or mutant DNA, such as circulating cell-free DNA (cfDNA) extracted from blood (liquid biopsy).
- Small insertions and deletions (indels) frequently occur in mutant *EGFR* (exon 19 deletion, exon 20 insertion) and *HER2* (exon 20 insertion). Although a well-designed PCR method may detect most of the indels, relatively uncommon ones that have the same pathologic effect are usually not covered. A fragment size analysis or sequencing based method is preferred; the latter could provide detailed information of the nucleotide changes.
- Fusions genes resulting from chromosome rearrangements (*ALK1*, *ROS1*, *RET*, *NTRK*, rarely *BRAF* and *FGFR*) are detectable by fluorescence in situ hybridization (FISH) with excellent sensitivity and specificity, if the probes are designed appropriately (see case 5 as an example). Because these fusion genes are all transcribed to mRNA with fusion protein expression, mRNA-based reverse transcription (RT) PCR is also an excellent detection method, providing better analytic sensitivity than FISH. However, RT-PCR may not cover all the translocation variants because rare or novel variants are usually not included in the primer design.

Table 7.2 Molecular genetic methods used in pathologic characterization of NSCLC

Method	Major features	Examples
FISH	Specific probes to detect translocations and amplifications; usually 200 cells scored Relatively labor intensive and not easily automated	<i>ALK1</i> and <i>ROS1</i> rearrangements, <i>MET</i> amplification
Polymerase chain reaction (PCR)		
RT-PCR	Identify abnormalities in mRNA, testing for fusion RNA and aberrant splicing	<i>EML4-ALK1</i> fusion, <i>MET</i> exon 14 skipping
Mutant allele specific PCR	Excellent in detecting SNVs, MNVs and small indels Need specific primer/probe design for each mutation	<i>EGFR</i> point mutations (L858R, T790M), and some indels such as exon 19 deletions
Droplet digital PCR	Same as allele-specific PCR, excellent analytic sensitivity, best for low tumor/mutant percentage	cfDNA test (liquid biopsy)
Fragment size analysis	Identify indels only Analytic sensitivity ~5%	<i>EGFR</i> exon 19 deletion <i>HER2</i> exon 20 insertion
SNaPshot	PCR-based, multiplex with variable probe lengths Only detects SNVs	<i>EGFR</i> (SNVs), <i>BRAF</i> V600E <i>KRAS</i> codon 12 and 13 mutations
Sequencing		
Sanger sequencing	Read the nucleotide sequence of a fragment up to 1000 bp; analytic sensitivity (~20%) not optimal for specimens with low tumor content Low throughput	Confirmation of germline variants and novel fusions, or other abnormalities when tumor content is sufficient
Pyrosequencing	Similar to Sanger sequencing; better analytic sensitivity (~5%), shorter sequencing length. Not optimal for regions with homopolymers	<i>EGFR</i> (SNVs and indels) <i>BRAF</i> V600E <i>KRAS</i> codon 12 and 13 mutations
NGS	High throughput, covering many genes/mutations in one test. Potentially detects a wide range of alterations. See discussion in question 12	DNA + RNA panel for tissue cfDNA testing (liquid biopsy)

Abbreviations: *FISH* fluorescence in situ hybridization, *SNV* single-nucleotide variant, *MNV* multinucleotide variant, *RT-PCR* reverse transcription PCR, *cfDNA* cell-free DNA, *NGS* next-generation sequencing

- Targetable gene amplifications in lung cancer occur in *MET*, *HER2*, and *FGFR*. The detection of gene amplification is challenging by PCR or sequencing methods. FISH with a chromosome enumeration probe (CEP) as internal control is a sensitive and specific method to detect gene amplification. For example, *MET* amplification is defined as a *MET/CEP7* (chromosome 7 enumeration probe) ratio >2.2. The probe design for specific genes is required, and a standardized cutoff threshold is critical to define an amplification. A copy number array analysis can sensitively detect gene amplification at the whole-genome level, but it is too costly to be routinely performed; only selected patients may be indicated.
 - More frequent than amplification, targetable alterations of *MET* include mutations at the RNA splice site or intronic region resulting in exon 14 skipping. Although many mutations have been confirmed resulting in exon 14 skipping, testing at the DNA level is challenging to recognize all the mutations to that effect [104]. Only an mRNA-based test (RT-PCR or RNA sequencing) could provide definitive evidence by amplifying the abnormally spliced mRNA with shorter length [105] (see case 6).
 - Although it is easier to implement FDA-approved in vitro diagnostic (IVD) tests [106], the laboratory directors need to have a full understanding of the detection range of the PCR-based tests to know whether additional testing would be required if the results from the IVD tests are negative.
 - Given the complexity and variable prevalence of the targetable genetic changes associated with NSCLC, there are different ways to approach the molecular testing in a clinical diagnostic laboratory [107]. It might be worthwhile for molecular diagnostic laboratories to establish a cost-effective test triaging an algorithm based on local resources and clinical requirements. Potentially practical algorithms include (1) test *KRAS* first, if any mutation identified, no other tests are indicated; (2) test *EGFR* first, if no mutation detected, reflex to *ALK1*, *ROS1* translocation, and/or *KRAS* mutation test, or a panel test to include more alterations. (3) start with a small panel to cover the most relevant genes, such as *EGFR* mutation and *ALK1* and *ROS1* translocations; if the results are all negative, then consider testing uncommon and investigational mutations with an expanded panel in a subgroup of patients as clinically indicated. In the future, most clinical laboratories will likely implement extensive large-panel NGS to cover all the mutations associated with NSCLC.
 - Laboratories performing mutation tests for NSCLC should have a plan for reflex testing in case any sample fails the first test or the quality/quantity not sufficient to complete the routine tests. In our institution, we perform a small panel (26 genes) NGS for SNVs/MNVs and small indels, together with a FISH panel including *ALK1*, *ROS1*, *RET* translocations, and *MET* amplification. If a sample fails to qualify for the NGS test, it is reflexed to a SNaPshot panel for the hotspot mutations of 10 genes including *EGFR*, *KRAS*, and *BRAF*, together with a fragment size analysis for *EGFR* exon 19 deletion, exon 20 insertion, as well as *HER2* exon 20 insertion. This approach allows us to obtain the mutation profile of all the clinically significant alterations from almost any sample, small or large.
 - In addition to molecular tests, immunohistochemical staining (IHC) for *ALK1* is considered as equivalent to FISH for ALK fusions; however, only the D5F3 antibody from Ventana has been approved by the FDA for patient selection for crizotinib treatment [106]. IHC can be used as a screening test for *ROS1* translocation; positive results must be confirmed by FISH [48]. *BRAF* V600E mutation can be screened by IHC with VE1 antibody; however, the performance characteristics need to be determined by the individual laboratory; and this antibody cannot detect *BRAF* non-V600E mutations. Due to the low prevalence of *HER2* amplification, the immunohistochemical stain of HER2 is not useful in predicting the treatment response of inhibitors or antibodies. IHC has no value in detecting *EGFR* mutations.
12. **What are the benefits and challenges of using next-generation sequencing (NGS)-based tests for non-small-cell lung cancer?**
- If designed appropriately, one NGS test can cover multiple genes, various types of mutations/aberrations, including point mutations, deletions, insertions, and copy number variants. Sequencing libraries built from tumor RNA can be loaded together with that from DNA to identify fusion genes and assess gene expression levels. TMB, a potential biomarker for immunotherapy, can also be calculated based on the data from a large-panel NGS (see question 15).
 - Other advantages of NGS include low input of genetic materials, high sensitivity, and low cost per gene. When multiple gene targets are tested for lung cancer, an NGS panel is more cost effective over multiple single-gene tests [107]. The high analytic sensitivity of deep sequencing coupled with molecular barcoding (unique molecular identifier, UMI) provides an ideal method to detect low-frequency mutant alleles, making it suitable for circulating tumor DNA (ctDNA) testing using blood samples (see answers to question 14 below).

- Because detecting *ALK1* and *ROS1* fusions are clinically required, designing a cost effective small NGS panel to cover all the clinically significant NSCLC alterations is challenging. The targeted panel NGS test can also be challenging in detecting large indels and copy number changes due to problematic alignment algorithms of bioinformatic pipelines for large indels, and the limited, sometimes biased, data available to calculate a normalized diploid level.
 - NGS panels, especially the large ones, generate a large amount of data. The post-sequencing analysis, variant interpretation, and data storage are current challenges for NGS tests. Furthermore, reporting many variants of uncertain significance (VUS) from a large panel may dilute the significance of clinically relevant mutations. Therefore, for the clinical mutation profiling of lung cancer, despite the increasing demand for larger panels from oncologists and patients, each molecular diagnostic laboratory needs to balance the panel size with the cost, turnaround time, and clinical relevance.
13. **What are the specimen types acceptable for mutation profiling of lung cancer?**
- Formalin-fixed, paraffin-embedded (FFPE) tissues or cell blocks are widely validated for FISH and molecular testing of NSCLC. The unstained FFPE sections for molecular tests should be unbaked. The routine 5- to 10- μ m thick sections are used, and the number of sections depends on the size of the tumor and the test to be performed. In general, five 5- μ m thick sections should be enough for non-sequencing assays, and ten 5- μ m sections may be preferable for NGS.
 - Fresh, frozen, or alcohol-fixed specimens are all acceptable for molecular tests; fresh or frozen specimens producing high-quality RNA are particularly good for RNA-based tests. However, each tissue type needs to be validated separately. Tissue samples treated with other methods such as acidic or heavy metal fixatives, or decalcifying solutions, should be avoided due to high false-negative rate [108–110].
 - Cytologic specimens including liquid-based cytology and fresh cell suspension, direct smear, and stained cytology slides can also be used for FISH and molecular tests. For cytology slides, Quick-diff is preferred than the Papanicolaou stain because DNA in the latter degrades faster [111].
 - Cellular fluids sampled from body cavities involved by metastatic tumors are usually sufficient for diagnostic evaluation, including molecular genetic testing. Peripheral blood, spinal fluid, and urine may be used to extract cfDNA for the assessment of therapy response and drug resistance in certain clinical circumstances.
14. **What is the role of cell-free DNA testing (liquid biopsy) for lung cancer?**
- Detecting cancer-related mutations from the circulating tumor DNA (ctDNA) shed into blood by apoptotic or necrotic tumor cells is referred to as “liquid biopsy.” Testing cfDNA from other body fluids can also be performed with the same methods [114]. As a minimally invasive procedure, liquid biopsy has quickly gained popularity among oncologists for NSCLC and other solid tumors.
 - Given the low concentration of cfDNA and variable fraction of ctDNA, a method with high analytic sensitivity is required for cfDNA-based cancer mutation detection. Currently most laboratories use droplet digital PCR or deep sequencing (NGS with high read depth) for cfDNA tests. The test protocols are challenging to validate and costly for each sample.
 - Studies have found excellent concordance and specificity (83.3–99.0%) of the mutation profiles detected from plasma compared with that from tissue samples. The clinical sensitivity of liquid biopsy ranges from 50% to 80% for guideline-recommended NSCLC biomarkers [48, 115–118].
 - It is assumed that mutations detected in ctDNA better reflect the heterogeneity of the tumors; therefore, mutation profiling with liquid biopsy could provide a more complete picture than testing tissue sampled from a single tumor region. On the other hand, the heterogeneous origin of ctDNA can complicate the result interpretation. A patient with lung cancer may have concurrent primary cancers in another organs; assigning a cfDNA mutation profile to lung cancer requires a complete evaluation of the patient to rule out other potential primary lesions.
 - Currently, there is not enough evidence to support the use of cfDNA molecular tests for the screening
- Regardless which tissue/sample type is used for FISH or molecular tests, the tumor content (the percentage of malignant cell nuclei) should be determined by a pathologist examining the corresponding cytology smear or H&E slides. A training and feedback program for pathologists evaluating the tumor cell content can significantly improve the skill and accuracy of estimation [112]. The analytic sensitivity (lower limit of detection, LLOD) of each test is method dependent. The current guidelines strongly encourage laboratories to implement tests that can detect mutations in specimens with as low as 10% cancer cells (5% mutant allele frequency) [48]. Circling the tumor-rich area on an H&E slide for macrodissection should be routinely performed to increase the tumor cell percentage and improve test accuracy, especially when the overall tumor cell percentage is low [113].

and diagnosis of primary lung adenocarcinoma. When there is already a diagnosis of NSCLC but the tissue sample is insufficient for molecular testing, cfDNA assay is an option to identify targetable mutations [48].

- When there is a need to identify the acquired resistant mutations, especially when it is clinically unfeasible to perform invasive procedures for tissue sampling due to comorbidities and/or other contraindications, a rebiopsy can be avoided if the cfDNA test is positive. Otherwise tumor sample testing should still be pursued due to the lower clinical sensitivity of cfDNA testing [48].
- Serial sampling to monitor the mutation evolution using cfDNA, which is much easier than repeat tissue biopsies, could reveal critical information to recognize the heterogeneous molecular basis of EGFR TKI [119, 120] or ALK inhibitor resistance [121, 122], especially when acquired *EGFR* T790M mutation decreases with the expanded use of third-generation EGFR TKIs [123].

15. What is the clinical significance of tumor mutation burden (TMB) in lung cancer and how is it tested?

- Tumor mutational burden (TMB) is a measurement of the total number of somatic mutations in the genome of tumor cells. It was originally defined as the number of nonsynonymous mutations per genome or per million base pairs (mut/Mb) calculated from the whole-genome (WGS) or whole-exon sequencing (WES) data [124]. The level of TMB is significantly variable across tumor types. NSCLC (both adenocarcinoma and squamous cell carcinoma) and SCLC have high average mutation burdens following melanoma [125, 126]. It is reasonably assumed that TMB can be a proxy for the number of neoantigens that is associated with the strength of immune response triggered by tumor cells.
- Multiple studies have shown that high TMB is associated with better responses to immune checkpoint inhibitors (ICIs) in NSCLC, independent of the expression levels of PD-L1 [47, 90, 127, 128]. TMB is, thereby, recognized as a powerful biomarker to select patients for ICI therapy although there are still some controversial results by other studies [129, 130].
- The gold standard for TMB was established from the data analysis of simultaneous WES of tumor and matched blood or normal tissue, in which only somatic nonsynonymous variants were included in the calculation. Recent studies demonstrated that data from NGS of a targeted panel with >300 genes could generate TMB results comparable to those calculated from WES [131–133].

- Multiple factors can affect the accurate assessment and interpretation of TMB [134]:

Preanalytical factors:

- FFPE tissue is most frequently used for NGS testing. Tissue quality and processing can significantly affect the level of artifacts, especially false-positive variants from nucleotide modifications.
- Tumor cell content and background tumor environment also affect the test results. Because a targeted panel sequencing can achieve higher sequencing depth than a WES, it is presumably more suitable for samples with lower tumor content that cannot be enriched (e.g., cytology cell block with relatively scattered tumor cells/clusters). However, this assumption has yet to be confirmed in clinical studies.
- Multiple studies have confirmed that TMB from plasma ctDNA correlates well with that from tissue samples (bTMB vs. tTMB [115, 135, 136]). If the value of bTMB is confirmed by correlating with clinical outcomes of immunotherapy, the utility of liquid biopsy could be largely expanded (see more discussion in question 14).

Analytical factors:

- The methods to calculate TMB and the cutoff value of high TMB need to be adjusted and validated based on the sequencing data from different platforms.
- To increase the accuracy of TMB measurement from targeted panel sequencing that generates less nonsynonymous variants, it is proposed to include synonymous variants and indels, which may render a better correlation with the TMB calculated from WES results.
- When a tumor sample is sequenced without paired normal tissue or blood sample, the germline variants can only be filtered through bioinformatic analysis using germline variant databases. Not all germline variants can be recognized this way; therefore, the TMB is likely overestimated.

Postanalytical factors:

- The procedure of interpreting and reporting TMB results have not been standardized, especially when the data from a targeted panel NGS is used.
- Currently there is no consensus cutoff value to define a high TMB. Although 10 mut/Mb is used as the threshold in the FDA approval of pembrolizumab for adults and children with TMB-H solid tumors [137], many WES-based studies reported TMB as total mutations per tumor. It is yet to be determined whether lung adenocarcinoma, SCC,

and SCLC need different TMB cutoff values to guide immunotherapy decisions.

- Before a consensus guideline is available for TMB reporting, the TMB Harmonization Consortium recommended that TMB be reported as mut/Mb [138]. The types of mutations included in the TMB calculation, the cutoff value, and the rationale to establish the cutoff should also be included in the report [139].

16. What other tests can be considered to provide guidance for immunotherapy of lung cancer?

- Assessment of PD-L1 expression by IHC is widely accepted as a useful marker to select NSCLC patients for immunotherapy with ICIs [140], although there are no standardized method and interpretation guideline, and the predictive value is questioned in some studies [141]. In addition to the TMB test discussed above, a combination of multiple biomarkers may provide a better prediction of ICI response in NSCLC patients.
- The mutation profiling routinely performed in most clinical laboratories for lung cancer has predictive value for ICI therapy. *EGFR* mutation, *ALK1*, or *ROS1* translocation-positive NSCLC usually responds poorly to ICIs even when the tumor expresses a high level of PD-L1 [142]. *TP53*-mutated, but *STK11* and *EGFR* wild-type NSCLCs are associated with longer disease-free survival (DFS) in ICI-treated patients [143].
- The evolution of ctDNA level in plasma defined by changes of the mutant allele frequency, regardless of specific mutations, may predict ICI response. Low ctDNA at the first evaluation after nivolumab treatment was associated with better clinical response, progression-free survival (PFS), and overall survival in a recent study [144]. The ctDNA level may become an early predictor of a durable good response to nivolumab [145].

While most studies on the efficacy of immunotherapy focus on the potential neoantigens created from genetic alterations of the malignant cells (the target), investigating the T-cells (the effector) mediating the killing of tumor cells have also provided information potentially valuable to independently predict responses to immunotherapy [146–148]. More clinical studies are required to confirm the validity of these results.

Case Presentations

The clinical histories of these cases are slightly modified to simplify the presentation and avoid the potential association of any protected patient information.

Case 1

Learning Objectives

- To understand the NGS test result of *EGFR* exon 19 deletion
- To discuss the nomenclature of complex variants detected by NGS

Case History

A 60-year-old female with a smoking history 30 years ago has a right upper lobe lung mass found at a routine clinic visit. A computerized tomography (CT) scan confirms a 3.7 × 2.4 cm single lesion. A core biopsy is performed and reveals lung adenocarcinoma. The mass is excised by a robotic right upper lobectomy with additional lymphadenectomy. Figure 7.1a, b show the histomorphologic findings are characteristic of invasive adenocarcinoma, acinar predominant; there is also in situ nonmucinous adenocarcinoma with a lepidic component shown in (b). Metastasis tumor is found in one ipsilateral lymph node.

Pathological Diagnosis

Adenocarcinoma, acinar predominant (acinar 55%, micropapillary 40%, papillary 5%), moderately to poorly differentiated

Maximum tumor diameter: 3.6 cm. The adenocarcinoma is staged IIB (T2a N1 M0)

Molecular Genetic Study

A 26-gene NGS test is performed on the lobectomy specimen. Tumor content is enriched by macrodissection. The only clinically significant alteration detected is shown in Fig. 7.1c. This is a complex sequence variant in exon 19 of *EGFR*, with a 15-base-pair deletion (codon 746 glutamic acid to 750 alanine) and a C to T variant at codon 751 Threonine. Based on the original data displayed in the image, the inframe deletion is on the same allele with the single nucleotide variant (*cis* mutations). It would not be entirely wrong to report the two variants separately as “*EGFR* E746_A750del (c.2236_2250del)” and “*EGFR* T751I (c.2252C > T)”; in fact, if the original alignment reveals the two variants are on different alleles (*trans* mutations), they must be reported separately. However, it is more appropriate to combine them as one mutation in the nomenclature to reflect that the complex variants are on the same allele:

- *EGFR* (NM_005228.3) c.2236_2252delinsAT (p.E746_751delinsI) in approximately 9% of alleles

Chromosome coordinates: chr7:55242466-55242482:GAATTAAGAGAAGCAAC > AT

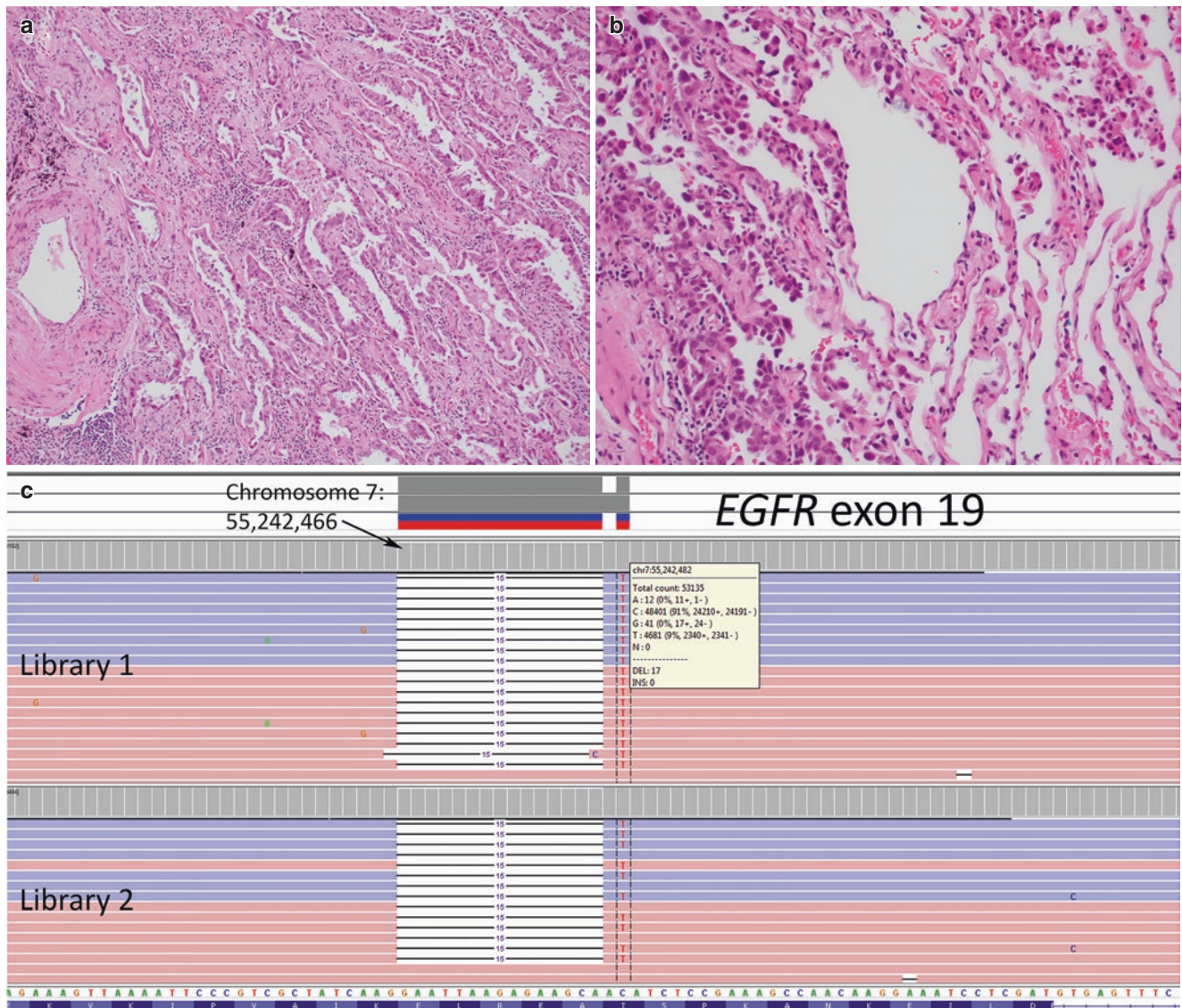


Fig. 7.1 Adenocarcinoma with *EGFR* exon 19 deletion. (a) and (b) Representative histomorphology images of the lobectomy specimen (H&E stain, A. 100X, B. 200X). (c) Screenshot of the NGS result displayed in the Integrative Genomics Viewer (IGV, Broad Institute) showing the *EGFR* exon 19 region. The NGS protocol leverages two separate libraries built from either strand of a DNA fragment (displayed on the upper and lower panel of image C, Library 1 and 2). The horizontal bars represent the sequencing reads, with purple and pink colors indicating

read directions. The chromosome coordinates indicated in the image are based on the reference assembly hg19, GRCh37. The reference DNA sequence and corresponding amino acids are displayed on the bottom. A 15-base-pair deletion and a C > T single nucleotide variant (SNV) are seen on the same sequencing reads in both libraries and both read directions. As displayed on the yellow box, the total variant frequency is 9% in this result with a total read depth of 53,135

Clinical Follow-Up

The patient receives adjuvant chemotherapy after surgery. No new or enlarging suspicious pulmonary nodules detected by follow-up CT scan 9 months later.

Discussion

Approximately half of the *EGFR* mutations in NSCLC are inframe exon 19 deletions, such as the mutation detected in this patient. These deletions result in increased kinase activity of EGFR, lead to hyperactivation of downstream proliferation and pro-survival signaling pathways, and conferred

increased sensitivity to EGFR tyrosine kinase inhibitor treatment.

As discussed above, the *EGFR* exon 19 inframe deletion in this case is complicated by an additional single-nucleotide variant. This specific variant is a known oncogenic variant described in TKI-responsive lung adenocarcinomas [149] and appears to be very similar to the well-characterized EGFR p. E746_A750del variant that confers TKI sensitivity in NSCLC.

The NGS result of this case signifies the importance for the molecular pathologists to review the original alignment data to generate an accurate mutation report.

Case 2

Learning Objective

The *EGFR* T790M mutation is the most common mechanism of acquired resistance after EGFR TKI-targeted therapy.

Case History

A 69-year-old male was diagnosed with bilateral metastatic adenocarcinoma of the lung with activating *EGFR* L858R mutation. He received targeted therapy using the first-generation TKI, erlotinib. Although he has no symptoms, based on imaging study results, the disease has slowly progressed in 2 years. A blood cfDNA test (liquid biopsy, performed by Guardant360® CDx) reported negative results; the original *EGFR* L858R mutation was not detected either. A biopsy of the right upper lobe lung lesion is performed.

Pathological Diagnosis

Before treatment (right lung transbronchial biopsy):

- Adenocarcinoma, non-mucinous with predominantly a lepidic growth pattern, intermediate nuclear grade

After 2 years of erlotinib treatment (right upper lobe, transbronchial biopsy):

- Adenocarcinoma, acinar-type growth pattern

Representative histomorphologic images of the two biopsies are shown in Fig. 7.2.

Molecular Genetic Studies

A 26-gene NGS test is performed on both biopsies.

Before treatment, two mutations detected:

- *EGFR* (NM_005228.3) c.2573T > G (p.Leu858Arg), 15% of alleles

- *TP53* (NM_000546.5) c.742C > T (p.Arg248Trp), 22% of alleles

After treatment, two new mutations detected:

- *CTNNB1* (NM_001098210.1) c.95A > C (p.Asp32Ala) 10% of alleles
- *EGFR* (NM_005228.3) c.2369C > T (p.Thr790Met), 9% of alleles; and c.2573T > G (p.Leu858Arg), 26% of alleles
- *TP53* (NM_000546.5) c.742C > T (p.Arg248Trp) 19% of alleles

Clinical Follow-Up

Erlotinib is discontinued after the second biopsy reported T790M mutation; treatment with third-generation EGFR inhibitor, osimertinib starts. Eight-weeks later, restaging CT scan shows about 50% size reduction in right upper lobe tumor and size reduction in scattered lung nodules. However, follow-up imaging scans 3 years later show the disease is still slowly progressing.

Discussion

Resistance to first- and second-generation EGFR TKIs can be either on-target that includes acquired EGFR resistance mutations or *EGFR* amplification or off-target that includes activation of non-EGFR pathways, lineage transformation, etc. *EGFR* T790M mutation is the most common on-target mechanism for the resistance of first- and second-generation EGFR TKIs. Molecular testing of T790M mutation is recommended for patients with disease progression while on EGFR TKI therapy, like in this case. Although liquid biopsy provides a minimally invasive way to detect additional mutations in these patients, the clinical sensitivity is relatively low. When a liquid biopsy result is negative, lesional tissue biopsy should be attempted. In this case, because the *EGFR* L858R mutation reported in the original biopsy sample was not detected by liquid biopsy, the negative result is problematic, and additional mutations are confirmed by follow-up tissue biopsy.

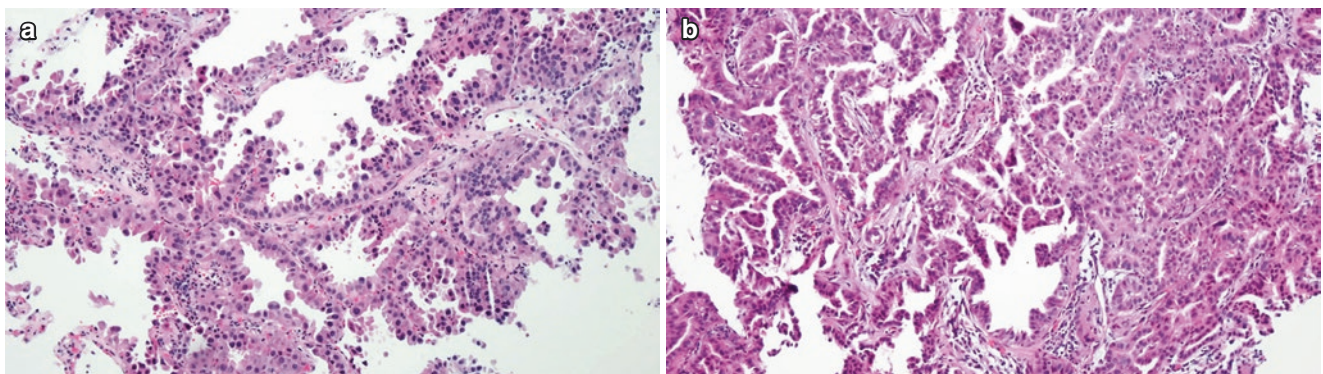


Fig. 7.2 Histomorphology of the adenocarcinoma biopsied before and after erlotinib treatment (H&E stain, 100X). (a) Before treatment: adenocarcinoma, primarily lepidic pattern; (b) After treatment: adenocarcinoma with a predominant acinar pattern

An additional *CTNNB1* p.Asp32Ala mutation is detected at the same allele frequency as *EGFR* T790M, further confirming the clonal evolution. It is not clear whether the *CTNNB1* and *TP53* mutations are associated with the patient's unsatisfactory response to the third-generation TKI therapy.

Case 3

Learning Objectives

- Synchronous multifocal lung cancers can be metastatic from one primary, or concurrent multiple primary lung cancers.
- Molecular profiling provides definitive clonal information for tumor samples. Combining histomorphologic and genotypic assessment is the ultimate approach to an accurate staging of lung cancer with multiple masses.

Case History

A 73-year-old female had a history of left breast cancer 20 years ago. A CT scan shows bilateral lung nodules. Three nodules located in the right lower lobe, middle lobe, and upper lobe are resected.

Pathologic Diagnosis

Right lower lobe (A):

- Adenocarcinoma, acinar type with lepidic pattern, maximum diameter 1.7 cm
- A separate nodule is consistent with metastatic carcinoma from breast (positively for GATA3 and mammaglobin; negative for TTF-1 and napsin)

Right middle lobe (B): Metastatic carcinoma from breast (based on morphology and IHC stains)

Right upper lobe (C):

- Adenocarcinoma, lepidic type (tumor size: 3.2 cm based on a CT scan report)

The histomorphologic features of the lung adenocarcinoma from block A2 and C1 are displayed in Fig. 7.3.

Molecular Genetic Studies

Only the lung cancer components (A2 and C1) are submitted for the 26-gene NGS test. The sequence variants detected are:

Right lower lobe (A2):

- *KRAS* (NM_033360) c.34G > T (p.G12C), 28% of alleles
- *APC* (NM_000038) c.3386T > C (p.L1129S), 46% of alleles

Right upper lobe (C1):

- *MET* (NM_001127500) c.3081_3082 + 2delAGGT (p.E1027fs), 14% of alleles; this deletion spans both exons and introns, most likely resulting in exon 14 skipping
- *APC* c.3386T > C (p.L1129S), 49% of alleles.

The two lesions are staged as separate primary tumors based on the molecular profiling results:

- Lower lobe stage: pT1b (1–2 cm)
- Upper lobe stage: pT2a: (3–4 cm)

Discussion

Multiple synchronous lung nodules are present in approximately 20% of the lung cancer cases [98, 99]. Determining whether they are independent primary tumors or intrapulmonary metastasis is crucial for the correct pathological staging and prognosis evaluation. Historically, the determination solely relied on histomorphologic features. Genetic studies can provide definitive evidence to determine the clonal relationship of different nodules. This patient presents with two separate lung adenocarcinomas at different lobes of the right lung, and the two tumor nodules share similar morphology. If one is primary and the other is metastasis, the stage will be pT4. In this case, the different driver mutations indicate they are two independent primary tumors; thus they are staged separately as pT1b and pT2a. Of note, the patient also has metastatic breast cancer nodules in the lung that are easily recognized by the different immunostaining patterns. If a metastatic cancer shows similar morphologic and immunostaining features with the primary lung cancer, it will be even more challenging to determine their origin without clonal evidence. A same *APC* sequence variant is detected in different primary lesions with an allele frequency close to 50%, suggesting this is most likely a germline variant. The *APC* p.L1129S is found in 0.10–0.24% of the general population (rs143638171). As a germline variant, it is considered benign or likely benign by contributors to NCBI's ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/RCV000077988/>). However, this variant is usually not filtered out as a benign germline variant due to its low prevalence in the general population. This kind of variant of uncertain clinical significance (VUS) is frequently encountered in cancer mutation profiling.

Case 4

Learning Objectives

- A small subset of lung carcinomas contains both adenocarcinoma and squamous components. Not all the different components can be sampled in a small biopsy.

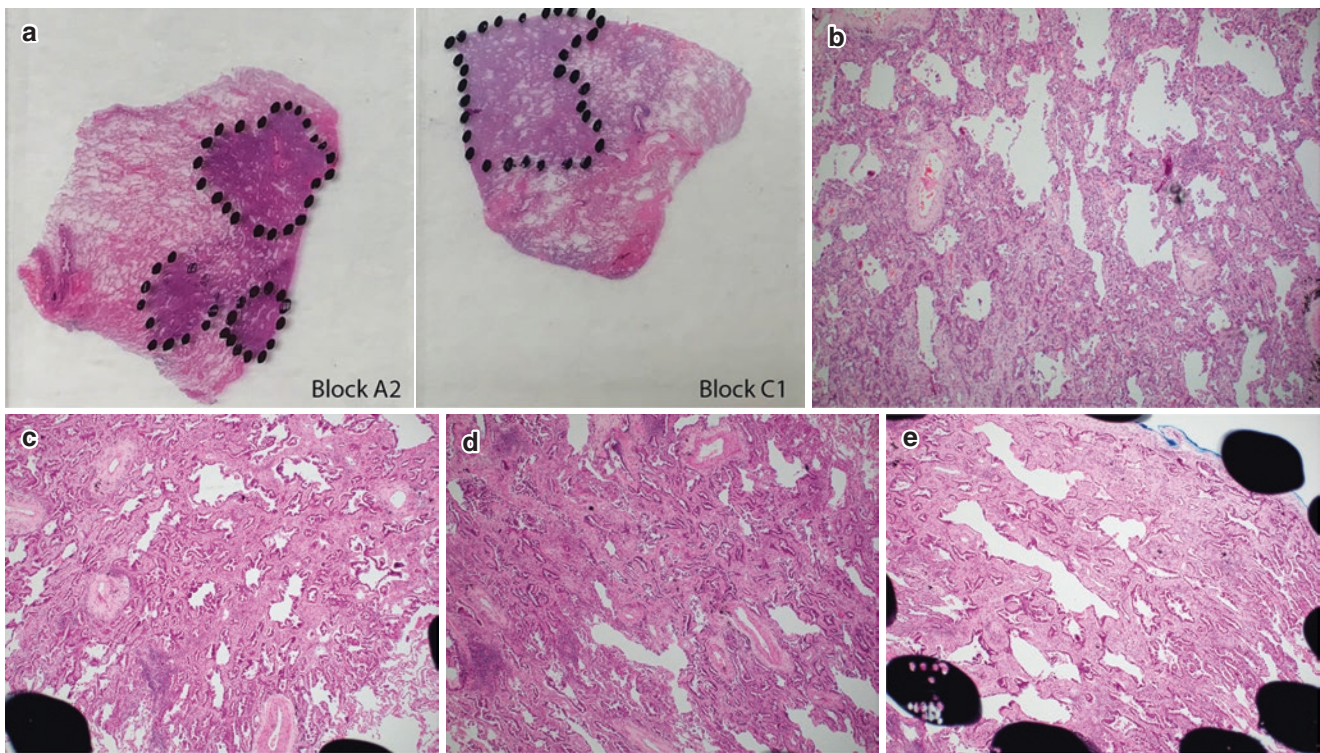


Fig. 7.3 Histomorphology of the adenocarcinomas from two different locations. (a) Photo taken directly on the glass slide of A2 (right lower lobe) and C1 (right upper lobe). These nodules have all been confirmed to be lung origin, not metastatic breast cancer, by IHC stains. Although three nodules are seen on slide A2, they partially merge with each other under microscopic observation. These nodules were not separated when

preparing for the NGS test. (b–e) Microscopic images, H&E stain, 40X. (b) Lesion from C1; (c, d, e) Three nodules from A2. These lesions show similar histomorphologic features of adenocarcinoma. It is unable to determine whether they are independent primary adenocarcinomas or metastasis from one primary

- Even if only squamous cell carcinoma is present in a core biopsy, it may be considered for molecular testing when there is a possibility of unsampled adenocarcinoma based on the clinical history or clues from histomorphologic features.

Case History

A 55-year-old female has a left femoral pathologic fracture. She was diagnosed with lung adenocarcinoma 2 years ago and treated with osimertinib based on the molecular test findings. A total hip arthroplasty is performed, and the bone specimen is received for pathology examination.

Pathological Diagnosis

Right lower lobe lung biopsy (from 2 years ago):

- Adenocarcinoma, lung primary

Left femoral neck resection:

- Metastatic keratinizing squamous cell carcinoma

The histomorphology images of both the lung and bone lesions are shown in Fig. 7.4.

Molecular Genetic Studies

A 26-gene panel NGS test performed on both specimens identifies the same mutation profiles (displayed in Figs. 7.5 and 7.6).

Lung mass:

- *EGFR* (NM_005228.3) c.2235_2249del (p.E746_A750del), 53% of alleles
- *APC* (NM_000038.5) c.3479C > A (p.T1160K), 58% of alleles

Bone (left femoral neck):

- *EGFR* (NM_005228.3) c.2235_2249del (p.E746_A750del), 48% of alleles
- *APC* (NM_000038.5) c.3479C > A (p.T1160K), 63% of alleles

Discussion

The histologic feature of the metastatic bone lesion is diagnostic of keratinizing squamous cell carcinoma, making it difficult to determine its primary origin. Mutation profiling with NGS identifies the lung and bone lesions share the same mutations, including the driver mutation

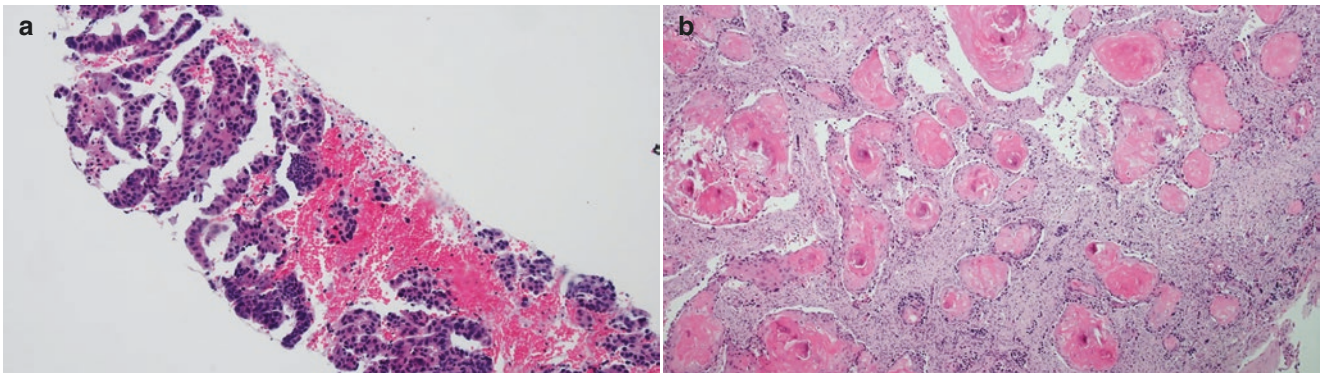
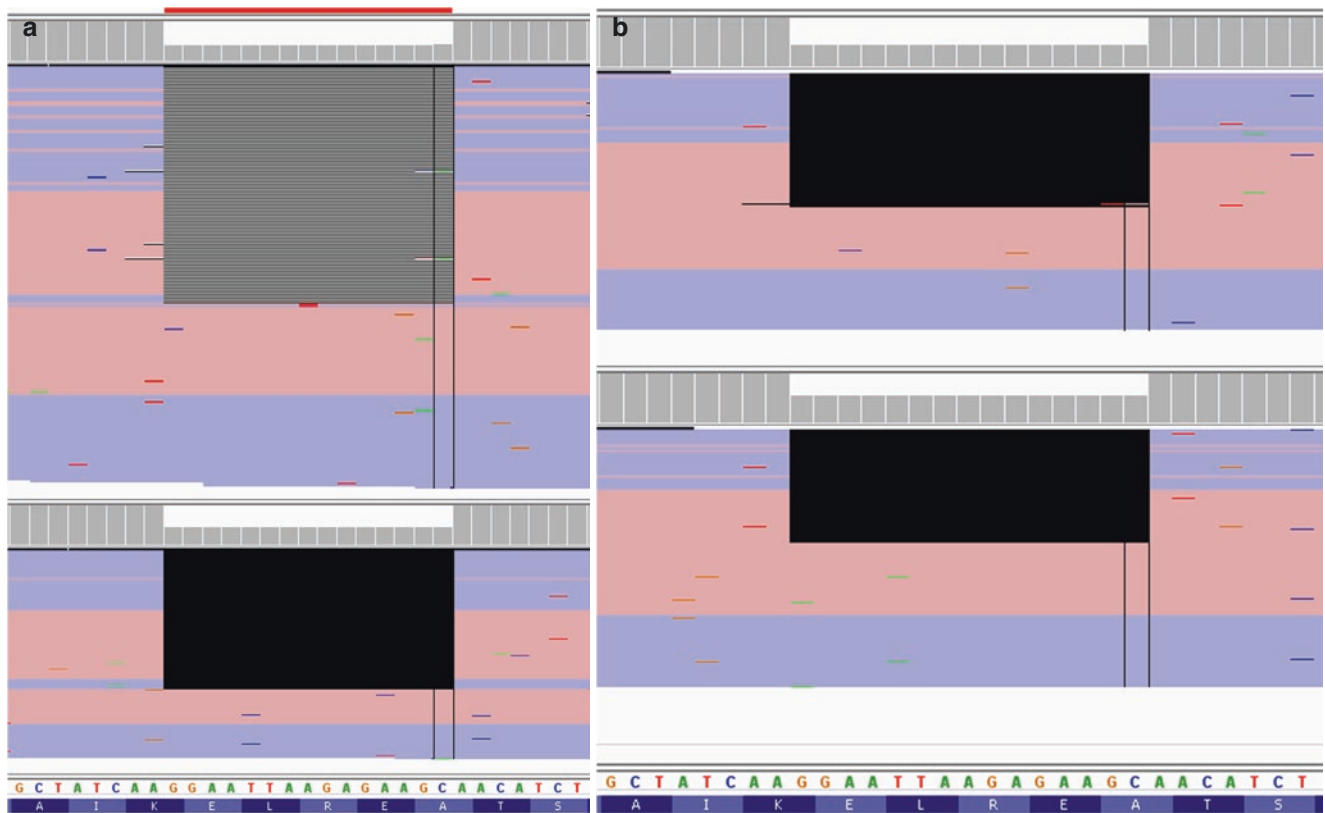
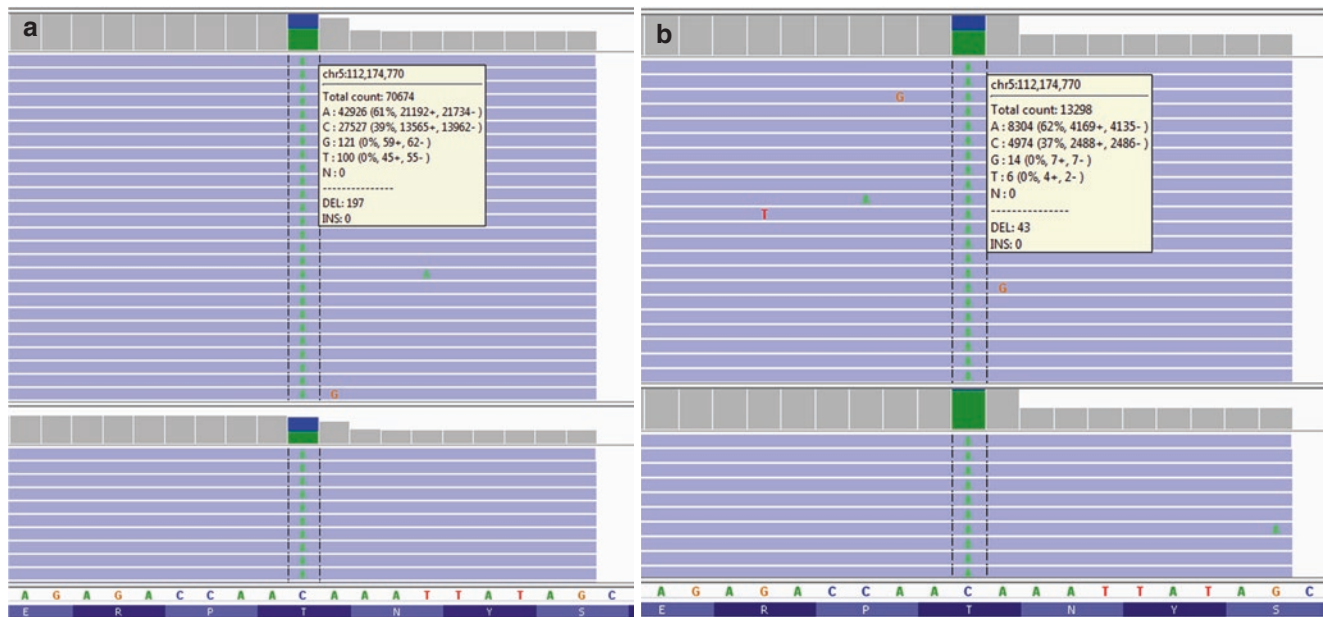


Fig. 7.4 Histomorphology of the primary and metastatic cancers. (a) Primary lung adenocarcinoma and (b) metastatic squamous cell carcinoma in the bone. (H&E stain, 100X)



Figs. 7.5 and 7.6 Screenshots of the NGS results displayed in IGV. The screenshots demonstrate that the same sequence variants are present in both (a) primary lung cancer and (b) metastatic bone lesion. The NGS protocol is the same as described in Fig. 7.1. Figure 7.5

EGFR exon 19 deletion; Fig. 7.6 *APC* c.3479 C > A (p.T1160K). More detailed information about the variant reads is displayed in the yellow box



Figs. 7.5 and 7.6 (continued)

EGFR exon 19 deletion, indicating the bone lesion is a metastasis of the NSCLC. In that regard, the results of molecular testing are useful in determining the origin of the metastatic tumor, especially when the morphologic features are different.

More importantly, this case demonstrates that various components may be present or derived from an NSCLC. We can reasonably hypothesize that a squamous cell carcinoma component was present in the primary lung cancer but not sampled in the biopsy.

Re-biopsy sampling for molecular test is indicated in patients whose lung cancer progresses or metastasizes to other locations while on targeted therapy to identify possible loss of actionable *EGFR* mutations or acquisition of resistant mutations, such as *EGFR* C797S, L718Q, G724S, and S768I. In this case, no other *EGFR* mutations were detected in the metastatic carcinoma in the bone.

Case 5

Learning Objectives

- To understand the FISH method detecting chromosome rearrangements that result in fusion genes
- To recognize the variant signal pattern of *ALK1* break apart FISH result

Case History

A 69-year-old male, non-smoker, is referred to ophthalmology for two right choroidal lesions. He is treated with steroids, but the lesions grew. Metastatic cancer is suspected, and a positron emission tomography (PET) scan shows a

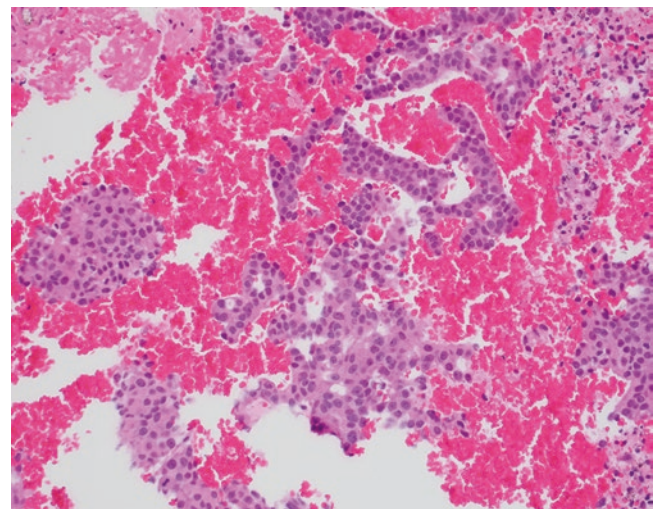


Fig. 7.7 Morphologic features of the tumor sample. Sections of the cell block made from the sub-carina lymph node fine needle aspiration sample showing malignant cells forming glandular architecture, consistent with lung adenocarcinoma. H&E stain, 200X

non-FDG avid nodule in the right lower lung lobe measuring 2.6×2.4 cm and a subcarinal lymph node measuring 1.9×2.0 cm with FDG 3.8. Fine needle aspiration of the subcarinal lymph node is performed.

Pathologic Diagnosis

The cytology smear and cell block show malignant cells (Fig. 7.7); immunostains performed on the cell block sections reveal the malignant cells are positive for TTF1 and napsin, consistent with metastatic adenocarcinoma from lung primary.

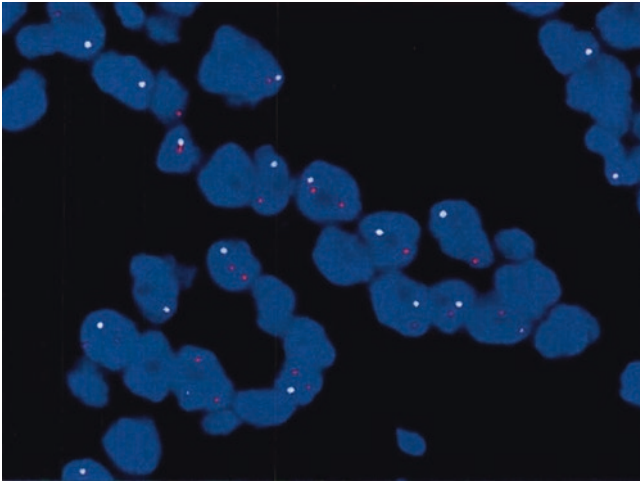


Fig. 7.8 Fluorescence in situ hybridization (FISH) of *ALK1* rearrangement. Dual-color break-apart probes show 1 fusion (red + green/yellow, the red part is smaller in many cells) and 1–2 red signals, indicating loss of 5' (green) signal. Although rearrangement typically shows split of green and red signals, loss of 5' green signal is a variant pattern indicating rearranged *ALK1* gene

FISH Analysis

- POSITIVE for variant *ALK1* gene rearrangement with loss of 5' *ALK* in 81% of the 200 cells scored (Fig. 7.8)

Discussion

Although *ALK1* rearrangements are present only infrequently in NSCLC, they are targetable alterations required to be tested per current practice guidelines. FISH is a sensitive and specific method to detect *ALK* rearrangements (and other rearrangements such as *ROS1* and *RET*). To cover variable partners of *ALK1* fusion, a break apart-probe design is preferred in the clinical FISH laboratory because the specific partner does not affect the therapy response. In the fusion protein, only the *ALK* kinase portion is functionally relevant; therefore a variant rearrangement with loss of 5' signal and retention of the 3' kinase portion, like in this case, is considered positive for *ALK1* fusion.

EML4 is the most common gene partner of *ALK1*; both genes are located in the short (p) arm of chromosome 2. Segment inversion of chromosome 2p results in the constitutively active *ALK-EML4* fusion protein. *AIK1* can also fuse with other genes, such as *TFG*, *KIF5B*, *KLC1*, etc. In this case, an *ALK-EML4* fusion gene has been confirmed in a later liquid biopsy test.

Case 6

Learning Objective

- Splice site mutation caused *MET* exon 14 skipping can be recognized at the DNA level in many cases.
- Definitive diagnosis of *MET* exon 14 skipping may require confirmatory tests at the RNA level.

Case History

A 70-year-old male is found to have a right upper lobe lung nodule (1.1 × 1.0 cm) and a subpleural left lung nodule (0.7 × 0.7 cm) in the CT scan during the work-up for his bladder cancer. A right upper lobe wedge resection is performed.

Pathologic Diagnosis

- Pulmonary adeno-squamous carcinoma (1.1 × 1.0 × 1.0 cm)

The histomorphology of this case and a control case is shown in Fig. 7.9a, d.

Molecular Genetic Studies

The 26-gene panel NGS test is performed on the sample after enrichment of tumor cells by macrodissection. A right upper lobe nodule biopsied from an 88-year-old female is used as a control for this case; the same molecular test is also performed on the control case.

The following mutation is detected in the patient sample:

- *MET* (NM_001127500) c.3082 + 1G > C, 16% of alleles, at chromosome 7: 116412044

The splice site mutation is likely to cause exon 14 skipping. A different single-nucleotide mutation is detected in the control case: *MET* c.3061T > C (p.Y1021H), 28.39% at Chromosome 7: 116412022. This variant should not affect RNA splicing, and the mRNA length should be the same as wild-type mRNA.

The NGS results are displayed in Fig. 7.9b, e.

RT-PCR amplification of the *MET* region flanking exon 14 is performed. *ABL1* Transcript serves as the amplification internal control. The results are deployed in Fig. 7.9c, f. The amplicon size from this patient (166 bp) is shorter than that from the control patient (281 bp, expected normal mRNA length).

Discussion

MET exon 14 skipping is a driver mutation in NSCLCs, and the absence of exon 14 leads to decreased *MET* protein degradation and increased kinase activity. RNA splicing requires a donor site (5' end of the intron), a branch site (near the 3' end of the intron), and an acceptor site (3' end of the intron). Mutations in these regions result in splicing error, leading to loss of exons or retraining of intron sequence. In this case, a G > C mutated splice donor site causes exon 14 skipping.

Although most mutations affecting RNA splicing can be identified by DNA sequencing and many have been confirmed by testing the mRNA, not all mutations causing *MET* exon 14 skipping are recognizable at the DNA level. A confirmatory RNA testing may be required if targeting *MET* active mutation is clinically indicated.

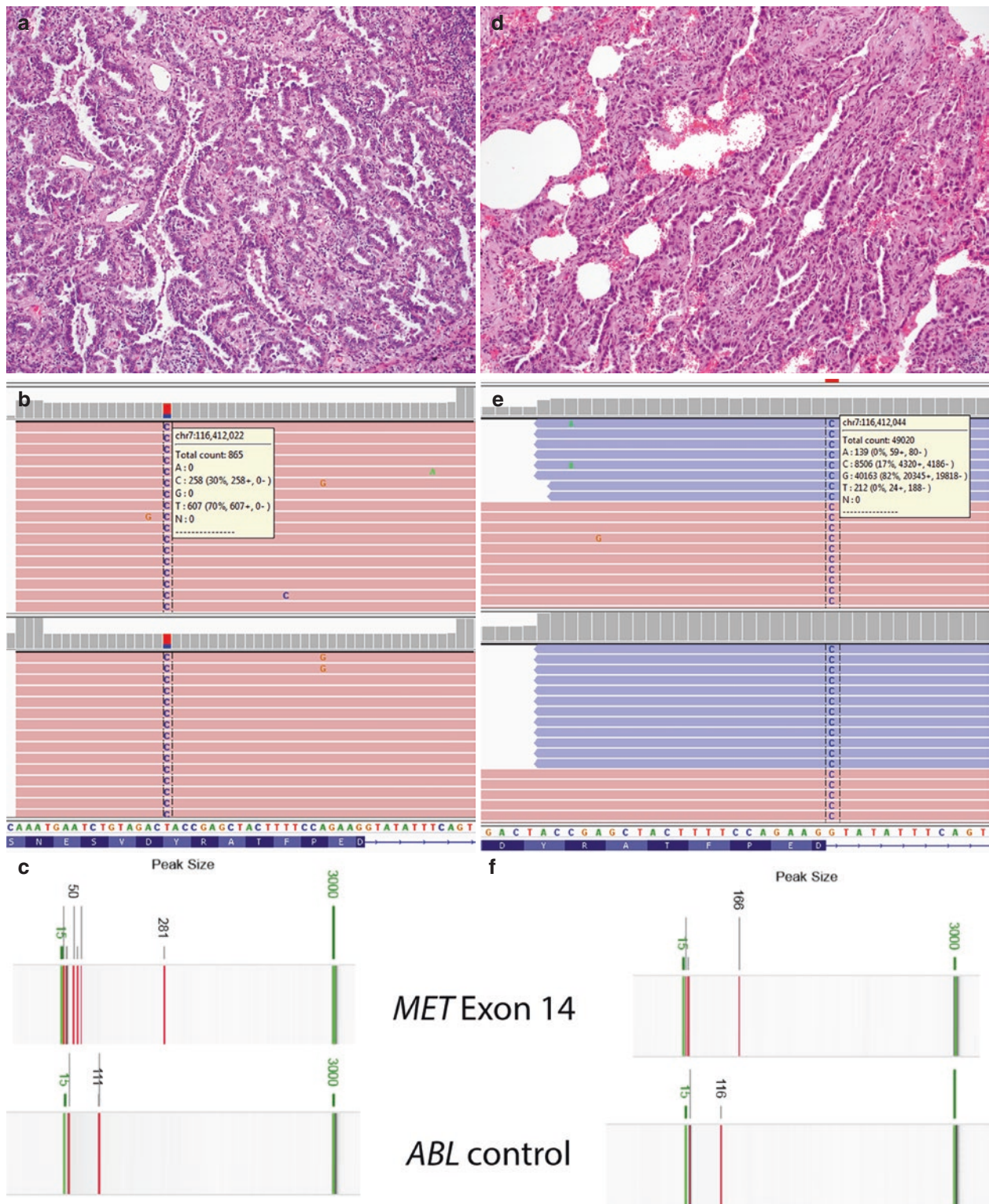


Fig. 7.9 Adenocarcinoma with *MET* exon 14 skipping mutation. This case (d–f) is illustrated in comparison to an adenocarcinoma with *MET* missense mutation (a–c). (a) and (d) Representative microscopic images, H&E stain, 100X. (b) and (e) IGV screenshots of NGS result. The NGS protocol is the same as described in Fig. 7.1. Displayed are the 3' region of *MET* exon 14 and the 5'-region of intron 14–15. Of note, the *MET* c.3061 T > C (p.Y1021H) in the control case has only one read direction due to the limited read length, but both libraries 1 and 2 reveal

the same variant (b). The *MET* exon 14 skipping is confirmed by a reverse transcription polymerase chain reaction (RT-PCR); *ABL* gene is used as the internal control for RT-PCR amplification. (c and f) PCR amplicons revealed by Qiaxcel® (Qiagen) capillary gel electrophoresis. A shorter amplicon is detected in the exon 14 skipping sample (166 bp in f vs. 281 bp in c). The expected *ABL* control amplicons (111/116 bp) are present in both cases

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