

Molecular biomarker testing for non–small cell lung cancer: consensus statement of the Korean Cardiopulmonary Pathology Study Group

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Molecular biomarker testing is the standard of care for non–small cell lung cancer (NSCLC) patients. In 2017, the Korean Cardiopulmonary Pathology Study Group and the Korean Molecular Pathology Study Group co-published a molecular testing guideline which contained almost all known genetic changes that aid in treatment decisions or predict prognosis in patients with NSCLC. Since then there have been significant changes in targeted therapies as well as molecular testing including newly approved targeted drugs and liquid biopsy. In order to reflect these changes, the Korean Cardiopulmonary Pathology Study Group developed a consensus statement on molecular biomarker testing. This consensus statement was crafted to provide guidance on what genes should be tested, as well as methodology, samples, patient selection, reporting and quality control.

Key Words: Carcinoma, non-small-cell lung; Biomarker; Precision medicine; Consensus

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Since the IRESSA Pan-Asia Study demonstrated that epidermal growth factor receptor (*EGFR*) mutations are a predictive biomarker for response to *EGFR* tyrosine kinase inhibitor (TKI) therapy in non–small cell lung cancer (NSCLC) patients, biomarker testing has become the standard of care for NSCLC patients [1]. Currently, various targeted drugs and their predictive biomarkers have been approved by the Korea Ministry of Food and Drug Safety (MFDS) and U.S. Food and Drug Administration (FDA) (Table 1).

The Korean Cardiopulmonary Pathology Study Group (KCPSG) has developed molecular pathology guidelines to respond to these challenges [2-5]. In 2017, the KCPSG and the

Korean Molecular Pathology Study Group (KMPSG) jointly published a guideline for molecular testing, including almost all known genetic changes that aid in treatment decisions or predict prognosis in patients with NSCLC [4]. Since then, major changes have been made to targeted therapies and molecular tests, such as newly approved targeted drugs or liquid biopsies. In order to reflect recent changes, the KCPSG has crafted a consensus statement to address issues of which genes should be tested, methodology, samples, patient selection, reporting and quality control.

The purpose of this consensus statement is to provide standardized guidelines for molecular biomarker testing to help se-

Table 1. Consensus statement of the Korean Cardiopulmonary Pathology Study Group

1. Which genes should be tested in non-small cell lung cancer patients in Korea?
EGFR, ALK, ROS1, and BRAF tests must be performed. NTRK, MET, RET, HER2, and KRAS tests are recommended when the results of EGFR, ALK, ROS1, and BRAF tests are negative or as part of broad testing panels.
2. Which testing method should be used?
Pathologists should use appropriate testing methods approved by Ministry of Food and Drug Safety for biomarker test.
3. Which samples can be used for molecular testing?
Any adequate tissue and cytology samples are acceptable for molecular testing. Liquid biopsy can be used when tissue is insufficient or not available for EGFR mutation test. If plasma test is negative, tissue biopsy is recommended.
4. What samples are adequate for molecular testing?
The minimum tumor cell content for proper analysis should be determined according to the analytic sensitivity of the testing method. Pathologist should pay attention to maximizing tumor cell content and the quality of nucleic acids for proper analysis.
5. Which patients should be tested?
Molecular testing for targetable alterations should be performed in all patients with non-small cell lung cancer.
6. How should the results be reported?
Reporting should follow the quality control guidance of the Korean Society of Pathologists and the Korean Institute of Genetic Testing Evaluations.
7. How should quality control be performed?
Internal and external quality control programs should be regularly implemented in accordance with the regulations of the Korean Society of Pathologists and the Korean Institute of Genetic Testing Evaluations.

EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; ROS1, ros proto-oncogene 1 receptor tyrosine kinase; BRAF, serine/threonine-protein kinase B-raf; NTRK, neurotrophic tyrosine receptor kinase; MET, mesenchymal epithelial transition; RET, rearranged during transfection; HER2, human epidermal growth factor receptor 2; KRAS, kirsten rat sarcoma virus.

lect NSCLC patients for targeted therapy in Korea. The KCPSG Molecular Testing Working Group reviewed and assessed existing guidelines developed by the KCPSG/KMPSG [4], the College of American Pathologists (CAP)/International Association for the Study of Lung Cancer (IASLC)/Association for Molecular Pathology (AMP) [6], the American Society of Clinical Oncology (ASCO) [7], and the National Comprehensive Cancer Network (NCCN) [8]. The workgroup endorsed most recommendations within the existing guidelines, but made minor modifications based on recent changes in targeted therapy as well as current Korean medical system (Table 1).

WHICH GENES SHOULD BE TESTED IN NON-SMALL CELL LUNG CANCER PATIENTS IN KOREA?

EGFR, ALK, ROS1, and BRAF tests must be performed; NTRK, MET, RET, HER2, and KRAS tests are recommended when the results of EGFR, ALK, ROS1, and BRAF tests are negative or as part of broad testing panels

The important oncogenic drivers in NSCLC are mutations of EGFR, anaplastic lymphoma kinase (ALK), ros proto-oncogene 1 receptor tyrosine kinase (ROS1), serine/threonine-protein kinase B-raf (BRAF), neurotrophic tyrosine receptor kinase (NTRK), mesenchymal epithelial transition (MET), rearranged during transfection (RET), human epidermal growth factor receptor 2 (HER2), and kirsten rat sarcoma virus (KRAS) [9]. Of these, sensitizing EGFR mutations, ALK and ROS1 fusions, BRAF V600E mutations, NTRK fusions, MET exon 14 skipping mu-

tations, and RET mutations or fusions, all have targeted drugs approved by the MFDS or FDA in NSCLC (Table 2). Targeted drugs for HER2 mutations are currently being investigated in clinical trials [10].

Sensitizing EGFR mutations occur within the kinase domain in exons 18 to 21. In particular, exon 19 deletions and exon 21 substitutions such as L858R account for about 90% of EGFR sensitizing mutations [9]. EGFR mutations occur in 37% to 40% of adenocarcinoma [11-13] and 9% of squamous cell carcinoma in Korea [13]. T790M is the most common resistance mechanism to EGFR TKI and occurs in 43%–50% of patients with acquired resistance to gefitinib/erlotinib [14]. Third-generation EGFR TKIs such as osimertinib inhibit both T790M and EGFR sensitizing mutations. Afatinib, erlotinib, gefitinib, and osimertinib have received reimbursement-approval by MFDS and National Health Insurance Service (NHIS) for NSCLC patients with EGFR mutations.

ALK fusions occur in about 5% of NSCLC patients, primarily in young never-smokers with adenocarcinoma [3,9,15]. More than 20 different ALK fusion partners have been discovered, of which EML4-ALK is the most common fusion protein in NSCLC [3,9,15]. Alectinib, brigatinib, ceritinib, and crizotinib have received reimbursement-approval for NSCLC patients with ALK fusions.

ROS1 fusions are reported in 1%–2% of NSCLC patients, primarily in young never-smokers with adenocarcinoma [9]. ROS1 is related to the ALK and insulin receptor superfamily. Because of high sequence homology to ALK, several ALK TKIs harbor dual inhibitory activity against ALK and ROS1 [9]. Crizo-

Table 2. Targetable genetic alterations in non-small cell lung cancer patients

Gene	Alteration	Method	Approved drug
EGFR	Mutation (Ex21L858R, Ex19del, Ex18, Ex20)	RT-PCR, NGS (approved commercial test: PANAMutyp R EGFR ^a , Cobas EGFR Mutation Test v2 ^a , GenesWell ddEGFR Mutation Test, Oncomine Dx Target Test ^b)	Afatinib, erlotinib, gefitinib, osimertinib
ALK	Fusion	Immunohistochemistry, FISH, NGS (approved commercial test: Vysis ALK Break Apart FISH, ALK D5F3 CDx)	Alectinib, brigatinib, ceritinib, crizotinib
ROS1	Fusion	Immunohistochemistry ^c , FISH, RT-PCR, NGS (approved commercial test: AmoyDx ROS1 Gene Fusions Detection Kit, ROS1 SP384 Assay ^c , Oncomine Dx Target Test ^b)	Crizotinib
BRAF	V600E mutation	RT-PCR, NGS (approved commercial test: PNAClamp BRAF Mutation Detection kit ^b , Oncomine Dx Target Test ^b)	Dabrafenib+trametinib
NTRK	Fusion	Immunohistochemistry ^c , NGS (approved commercial test: pan-TRK EPR17341 Assay ^c)	Larotrectinib ^d , Entrectinib ^d
MET	Exon 14 skipping mutation	NGS	Crizotinib ^e , capmatinib ^f , tepotinib ^f
RET	Fusion, mutations	NGS	Selpercatinib ^f
HER2	Mutation (Ex20ins)	NGS	Trastuzumab ^e
TMB		NGS (approved commercial test: FoundationOne CDx ⁱ)	Pembrolizumab ^f

EGFR, epidermal growth factor receptor; RT-PCR, real time polymerase chain reaction; NGS, next generation sequencing; ALK, anaplastic lymphoma kinase; FISH, fluorescent in situ hybridization; ROS1, ros proto-oncogene 1 receptor tyrosine kinase; BRAF, serine/threonine-protein kinase B-raf; NTRK, neurotrophic tyrosine receptor kinase; MET, mesenchymal epithelial transition; RET, rearranged during transfection; HER2, human epidermal growth factor receptor 2.

^aApproved for tissue and plasma; ^bApproved as new health technology; ^cFor screening; ^dNon-reimbursement approval; ^eApproval of non-reimbursement use of drugs exceeding the scope of product approval; ^fNot approved in Korea (as of March 17, 2021).

tinib has received reimbursement-approval for NSCLC patients with *ROS1* fusions.

BRAF is a serine/threonine kinase downstream of *KRAS* in the mitogen-activated protein kinase (MAPK) signaling pathway. *BRAF* mutations occur in 1%–2% of NSCLC patients and are mostly represented by V600E [9]. Specific clinical or pathological features associated with *BRAF* mutations have not been defined. Compared to *BRAF* inhibitor monotherapy, treatment with combination *BRAF* and MEK inhibitors improved response rates and progression free survival due to delays in MAPK-driven acquired resistance as well as reduced toxicities from paradoxical MAPK pathway activation [8,9]. The combination therapy with *BRAF* and MEK inhibitors has received reimbursement-approval for NSCLC patients with *BRAF* V600E mutations.

NTRK encodes for three transmembrane proteins, TRKA, TRKB, and TRKC, which play an important role in central nervous system development and maturation [8,9]. *NTRK* fusions have been identified in several types of solid tumors and occur in less than 1% of NSCLC patients [8,9,16]. Larotrectinib received non-reimbursement-approved for the *NTRK*-positive solid tumors including NSCLC.

Genetic alterations in *MET* such as exon 14 skipping mutations, amplification, and protein overexpression, are functionally important in cell proliferation, migration, and invasion [8,9]. *MET* exon 14 skipping mutations occur in 3%–4% of adenocarcinoma and 1%–2% of other NSCLC histologies [8,9]. TKIs targeting *MET* are subdivided into multikinase such as crizo-

tinib and selective *MET* inhibitors such as tepotinib and capmatinib. Crizotinib, tepotinib, and capmatinib were granted breakthrough therapy status in 2018, 2019, and 2020, respectively, for the treatment of NSCLC patients with *MET* exon 14 skipping mutations by FDA. In Korea, crizotinib received approval for non-reimbursement use of drugs exceeding the scope of product approval.

RET fusions occur in 1%–2% of NSCLC and are more frequent in young never-smokers with adenocarcinomas [8,9]. Selpercatinib was granted breakthrough therapy status for the treatment of NSCLC patients with *RET* fusions by FDA in 2020. In Korea, ceritinib received approval for non-reimbursement use of drugs exceeding the scope of product approval.

HER2 amplification and mutations are found in up to 4% and 35%, respectively, of NSCLC [9]. Most *HER2* mutations occur in exon 20 and lead to uncontrolled signaling activation through the same pathways activated by *EGFR* [9]. To date, no specific *HER2* inhibitors have been approved and trials targeting *HER2* exon 20 mutations or gene amplification have been investigated [8,9]. In Korea, trastuzumab received approval for non-reimbursement use of drugs exceeding the scope of product approval.

KRAS mutations occur in about 25% of NSCLC [8,9]. *KRAS* mutations are mutually exclusive to other oncogenic driver mutations and allow the identification of patients who are unlikely to have targetable alterations [8,9]. Studies aimed at exploring effective targeted therapies for *KRAS* mutations have been performed, with a recent clinical trial for *KRAS* G12C inhibitors

showed promising results in preclinical and clinical settings [17]. The predictive role of *KRAS* mutations for response to chemotherapy, targeted therapy, anti-vascular therapy, or immunotherapy is currently controversial [17]. It is impractical to perform multiple single gene tests for all oncogenic drivers due to limited samples and as well as the financial burden on both patients and healthcare insurance. The 2018 CAP/IASLC/AMP guideline recommended that tests for *EGFR*, *ALK*, and *ROS1* must be offered by all laboratories as an absolute minimum [6]. *BRAF*, *MET*, *RET*, *HER2*, and *KRAS* should be included in any expanded panel if adequate material is available. All other genes can be considered for clinical trials at the time of publication. The 2018 ASCO guideline endorsed the CAP/IASLC/AMP guideline but added *BRAF* in the absolute minimum category [7]. This is because dabrafenib/trametinib combination therapy for NSCLC with *BRAF* v600E mutations was approved in 2017 by FDA [6], just after the 2018 CAP/IASLC/AMP guideline panel completed its literature review [7,18]. The 2020 NCCN guideline also recommended tests for *EGFR* (category 1), *ALK* (category 1), *ROS1* (category 2A), and *BRAF* (category 2A) [8]. The 2020 NCCN guideline added tests for *MET* (category 2A) and *RET* (category 2A) based on recent data showing the efficacy and FDA approval of corresponding TKIs [8]. For *HER2*, *KRAS*, and *NTRK*, NCCN guideline strongly advised broad molecular profiling (category 2A) [8].

The extent of what constitutes the absolute minimum of predictive biomarkers should be decided by the approval and reimbursement status of the corresponding targeted therapies in each country. In Korea, targeted drugs for NSCLC patients with *EGFR* mutations, *ALK* fusions, *ROS1* fusions, or *BRAF* V600E mutations received reimbursement-approval as of October 15, 2020 (Table 1). Thus, we recommend *EGFR*, *ALK*, *ROS1*, and *BRAF* tests must be performed for treatment decision in NSCLC patients. Targeting drug for *MET* exon 14 skipping mutations, *RET* fusions, and *HER2* mutations can be used only as non-reimbursement use of drugs exceeding the scope of product approval in NSCLC patients. *NTRK* TKI is approved as non-reimbursement. Therefore *MET*, *RET*, *HER2*, *KRAS*, and *NTRK* tests are recommended when the results of *EGFR*, *ALK*, *ROS1*, and *BRAF* tests are negative or as part of broad panels. Molecular testing for other genetic alterations can be performed for clinical trials.

Although not an oncogenic driver, tumor mutational burden (TMB) is an emerging biomarker for selection of patients with NSCLC for immunotherapy. TMB is defined as the number of mutations per megabase (Mb) of DNA [6,8,19]. In NSCLC,

smoking exposure and the associated genomic profile contribute significantly to high TMB [20]. Mutant proteins derived from somatic mutations produce neoantigens. Tumors with more neoantigens could elicit a stronger CD8 T-cell response in the presence of immunotherapy agents [20]. Therefore, TMB is considered a surrogate marker of neoantigen burden and therefore a predictive biomarker for immunotherapy. Several clinical trials have investigated tissue or blood based TMB as a predictive biomarker for immunotherapy [19]. The KEYNOTE-158 trials showed a higher response rate for patients with high TMB, which was defined as ≥ 10 mutations/Mb on a FoundationOne CDx assay (Foundation Medicine, Cambridge, MA, USA) to pembrolizumab [19,21].

WHICH TESTING METHOD SHOULD BE USED?

Pathologists should use appropriate testing methods approved by MFDS for biomarker test

It is important to consider the clinical utility, status of approval, and reimbursement in determining testing methods (Table 1). For *EGFR* testing, the CAP/ASCO/IASLC guideline recommended that testing methods must be able to detect molecular alterations in specimens with as little as 20% cancer cells. Furthermore, assays capable of detecting abnormality in as little as 5% tumor cells should be used for *EGFR* T790M mutation [6]. Recently developed polymerase chain reaction (PCR)-based methods such as real time PCR (RT-PCR) and digital PCR (dPCR) are more sensitive than Sanger sequencing and can reliably detect low frequency mutations in samples with as little as 5%–10% cancer cells. In Korea, peptide nucleic acid clamping RT-PCR (Panagene, Daejeon, Korea), Cobas EGFR Mutation test (Roche, Indianapolis, IN, USA), pyrosequencing, and next generation sequencing (NGS) are commonly used for *EGFR* mutation test. Recently, droplet digital PCR (ddEGFR mutation test, Gencurix Inc., Seoul, Korea) and targeted NGS panel (Thermo Fisher Scientific, Waltham, MA, USA) have been approved as new health technologies. Mutation specific immunohistochemistry (IHC) is not encouraged for detection of *EGFR* mutations because it has low sensitivity and recent advances in molecular technology enable the detection of mutations in limited amounts of sample [6,8]. *EGFR* gene amplification or total protein expression should not be used to select patients for *EGFR* TKIs [6].

For *ALK* fusions, fluorescent in situ hybridization (FISH) and IHC with *ALK* D5F3 CDx assay (Ventana Medical Systems Inc., Tucson, AZ, USA) are recommended [6,8,22]. Only Vysis ALK Break Apart FISH (Abbott Molecular Inc., Abbott Park,

IL, USA) received approval for selection of NSCLC patients for treatment with brigatinib. The ALK D5F3 CDx assay has an overall sensitivity of 81%–100% and specificity of 91%–100%. The ALK CDx is an equivalent alternative to ALK FISH [6,8,22]. The ALK CDx assay is widely used due to low cost, shorter turnaround time, and ease of use. It is important to understand several potential pitfalls of ALK CDx interpretation [22]. False positive staining, often weaker than true positive expression, may be seen in alveolar macrophages, airway epithelial cells, extracellular mucin, and necrotic debris. Tumor cells with neuroendocrine differentiation may show false positive staining although their expressions are typically heterogeneous or in a checkerboard pattern [22]. Therefore, samples with focal or equivocal expression are recommended to be retested with ALK FISH.

For *ROS1* fusions, AmoyDx ROS1 gene fusions detection kit (Amoy Diagnostics Co., Xiamen, China) received approval for selection of NSCLC patients for crizotinib. FISH and NGS are available for detection of *ROS1* fusion but cannot be used for treatment decisions in Korea. *ROS1* IHC may be used as a screening test [8]. For *BRAF* V600E mutations, NGS and PNAClamp *BRAF* mutation detection kit (Panagene) can be used for treatment decision.

For other genetic alterations, the CAP/ASCI/IASLC guideline and NCCN guideline recommends that broad panels are preferred over multiple single gene tests to identify other treatment options [6,8]. We agree with this, and recommend NGS for *NTRK*, *MET*, *RET*, *HER2*, and *KRAS*. When NGS is not available, IHC may be used as a screening test for *NTRK* fusions and positive results should be confirmed by NGS [23]. NGS allows the assessment of many targetable genetic alterations from small samples at once [8,24]. Targeted NGS using amplicon resequencing enables the detection of point mutations with much higher sensitivity compared to single gene targeted tests [8,24]. NGS can detect gene fusions, in particular using an RNA-based approach [25]. Despite these advantages, NGS has not yet become a standard practice in Korea. NGS receives reimbursement only when performed by an approved medical institution. NGS is still more expensive than the sum of single gene tests for *EGFR*, *ALK*, *ROS1*, and *BRAF* as of 2020 in Korea. In addition, NGS is not approved for treatment decisions for NSCLC patients with *ROS1* or *ALK* fusions. A detailed laboratory guideline for NGS is beyond the scope of this paper (see Kim et al. [26] for guidelines and requirements for clinical NGS tests).

WHICH SAMPLES CAN BE USED FOR MOLECULAR TESTING?

Any adequate tissue and cytology samples are acceptable for molecular testing; Liquid biopsy can be used when tissue is insufficient or not available for *EGFR* mutation test; If plasma test is negative, tissue biopsy is recommended

The CAP, ASCO, and NCCN guidelines recommended that any tissue and cytology samples with adequate cellularity and preservation are suitable for molecular testing [6-8]. We agree this recommendation and encourage active utilization of cytology samples for molecular testing because patients with advanced lung cancer often cannot undergo tissue biopsy or, may have biopsies with insufficient tumor tissue.

Various cytology preparations including cell blocks, direct smears, cytopsin preparations, and liquid-based cytology are acceptable and reliable for biomarker testing [6-8]. Molecular testing results using cytology samples are highly concordant with those of the corresponding tissue samples, in particular with more sensitive methods [27-32]. A study comparing concurrently acquired fine needle aspiration and core needle biopsy samples showed better cellularity, higher tumor fraction, and higher mutation allelic frequencies in aspiration smears than biopsy samples [30]. It is important to understand the advantages and disadvantages of cytology samples for appropriate triaging of biomarker tests.

Formalin-fixed, paraffin-embedded (FFPE) cell blocks are widely used for molecular testing. The main advantage of cell blocks is to allow serial sections for multiple tests. Because most biomarker assays have been validated on FFPE samples, cell blocks do not require additional validation [33-38]. Compared to alcohol-fixed cytology samples, the limitations of cell blocks are due to formalin artifacts in DNA and nuclear truncation [33-36]. Formalin leads to cross-linking and chemical modification of nucleic acids which may affect DNA quality. In FISH, the presence of truncated nuclei in cellblock sections may result in artificial loss of probe signal, as seen in conventional FFPE block [3]. Cell blocks often exhibit depletion of tumor cells on deeper sections.

Air dried or alcohol-fixed cytology samples such as smears and cytopspins may offer higher quality nucleic acids than cell blocks [33-38]. Smears, cytopspins, and liquid-based cytology allow an evaluation of the adequacy and cellularity of tumor cells on site and the opportunity to scrape tumor cells by macro/microdissection [33-38]. They enable the presentation of whole tumor nuclei for FISH [3]. The main limitation of smears is the sacri-

fice of archival stained slides. Whole slide imaging may be used for archiving cytology slides. The use of non-FFPE cytology samples may require further validation of molecular testing. ALK CDx has not been approved for non-FFPE cytology samples.

Tissue-based molecular testing is often not available in NSCLC patients due to the invasiveness and high risk of complications of biopsy procedures or due to insufficient tumor tissue obtained [5,39]. In addition, failure rate for re-biopsy was reported to be about 20% in NSCLC patients with progression or metastasis [40]. The liquid biopsy is a non-invasive way to detect genetic alterations. Tumor biopsies are often not able to encompass comprehensive genomic profiles due to tumor heterogeneity. However liquid biopsy is more likely representative of whole tumor clones because tumor DNA is constantly released into the bloodstream from all tumor sites [39,41]. Studies using various analytes such as circulating tumor cells, circulating tumor DNA (ctDNA), tumor educated platelets and tumor-derived exosomes obtained from blood or other body fluids have shown promising results for detection of genetic alterations in NSCLC patients. But, plasma ctDNA assay is only approved by MFDS and FDA. Plasma ctDNA testing for detection of *EGFR* mutations to select patients for EGFR TKIs has been covered in Korea since 2018.

The limitation of liquid biopsy is that ctDNA level is often extremely low and requires highly sensitive techniques. Moreover, germline cell free DNA (cfDNA) contamination from white cells could dilute ctDNA levels. In meta-analysis studies, the sensitivity of plasma ctDNA assay was about 67% [6,42]. The sensitivity can be increased up to 72% and 87% by use of more sensitive methods such as dPCR and NGS, respectively [42]. The specificity of plasma ctDNA assay was 96% for overall mutations and 80% for T790M [6,42]. The relatively low specificity could be in part, due to the high tumor heterogeneity, which can lead to false negative results in tissue tests [42]. This is supported by results of clinical trials showing that plasma T790M-positive patients had similar outcomes to tissue T790M-positive patients for osimertinib treatment [43,44]. Therefore, plasma testing should be performed in patients whose tissue material is unavailable or insufficient. And if plasma test is negative, repeat biopsy should be done for *EGFR* testing and other genetic alterations.

WHAT SAMPLES ARE ADEQUATE FOR MOLECULAR TESTING?

The minimum tumor cell content for proper analysis should be determined according to the analytic sensitivity of the testing method. Pathologist should pay attention to maximizing tumor cell content and the quality of nucleic acids for proper analysis

Sample adequacy is determined by the quantity and quality of tumor cells. The minimum tumor cell content for mutational testing is largely dependent on the analytic sensitivity of the testing method. Recently developed RT-PCR and dPCR techniques require samples with a minimum of 10% tumor cell content. However, in an unpublished survey of KCPSG, half of respondents said they performed *EGFR* tests even when tumor cell content was less than 10% or there were less than 200 tumor cells. Various approaches for tumor saving and enrichment enable the detection of genetic alterations even in samples with a tumor content less than the platform threshold [45].

The most commonly used strategy for tumor enrichment is manual macro/microdissection [45]. Pathologists review hematoxylin and eosin (H&E) stained slides and mark tumor areas directly on the slide. Subsequently, the corresponding areas are manually scraped off from the slides or procured directly from the block using the marked H&E slide as a guide. The EURTAC trial showed similar analytic accuracy in samples with less than or equal to 10% tumor content with microdissection, compared to samples with more than 10% without microdissection for *EGFR* test [46]. Samples with more than or equal to 30% tumor content in the dissected area showed good success rates of 95.6%, regardless of the size of the dissected area [31].

In small samples such as needle biopsy or cell block, tumor cell content is often limited and disappears on deeper sections. Thus, tumor saving is important for biomarker testing. The most effective tumor saving strategy is the pathologist ordered reflex test, in which the pathologist orders a predefined set of biomarker tests at the time of pathologic diagnosis [45,47]. In reflex testing, predefined tests are performed all at once using pre-cut unstained slides. This avoids tissue waste due to trimming or refacing and increases the quality of molecular testing [45,47-49]. In addition, this standardized and comprehensive approach by the pathologist led to reductions in turnaround time and an increase in the number of patients with biomarker testing results available at the time of treatment decision [48,49]. The limitation of reflex testing is that early-stage NSCLCs would be included. Several trials for adjuvant and neoadjuvant target therapy in early-stage NSCLC

patients are ongoing with promising preliminary results [50,51]. The aura 3 trial showed longer disease-free survival among those who received osimertinib than among those who received placebo in patients with stage II to IIIA *EGFR* mutation–positive NSCLC [51].

The second strategy for tissue saving is to make one block for one core for needle biopsy specimen. The one core-one block strategy also avoids unnecessary trimming and allows representative cross-sections of all tissue cores embedded in a single block [45]. Multiple blocks are distributed to appropriate tests according to the tumor cell content of individual blocks, allowing efficient use of the tissue [45]. Finally, IHC to distinguish between adenocarcinoma and squamous cell carcinoma should be minimized in small samples. Large IHC panels may not provide an advantage over a limited immunohistochemical workup using thyroid transcription factor 1 (or Napsin A) and p40 (or p63) [8,52].

ALK or ROS1 FISH requires a minimum of 50 to 100 viable tumor cells based on the interpretation guideline and if the result is equivocal, an additional 50 nuclei should be evaluated by the second reader [4]. It is recommended to avoid areas of necrosis and areas where the nuclear borders overlap or are indistinguishable from the adjacent stromal cells. If the tumor is very focal within the sample, the area to be examined on the slide can be marked for easy identification with a dark field fluorescence microscope. The ALK D5F3 CDx can be performed on FFPE samples with any tumor cells.

The important preanalytical factors affecting nucleic acid and protein quality are cold ischemia time, total fixation time, fixatives, and decalcification [53,54]. One hour or less of cold ischemia time (time between specimen removal from the body and its stabilization in formalin) is recommended [53,54]. Cold ischemia time of less than 1 hour for FISH, less than 2 hours for protein and RNA, and 24 hours or less for DNA have been reported to be acceptable [53]. The recommended fixative is 10% neutral, phosphate-buffered formalin. The 10% neutral buffered formalin is the most commonly used fixative for routine histology, providing excellent morphological preservation. Most IHC and molecular tests are validated for FFPE samples. Total fixation time of 6 to 24 hours is recommended. Fixation time of 6 to 24 hours for protein, 8 to 48 hours for RNA, and less than 72 hours for DNA is acceptable [53,54]. Strict quality control for concentration and pH of formalin and total fixation time is required to minimize formalin artifacts that can affect nuclei acid [54]. Of liquid based cytology preservative, CytoRich Red containing small amounts of formaldehyde may affect DNA yield [55]. Ethylenediaminetetraacetic acid (EDTA) is recom-

mended for decalcification, in particular for needle biopsy and small samples [56,57]. EDTA preserves morphology and nucleic acid but slowly decalcifies. Decalcifying agents containing strong acids do not yield adequate DNA for molecular testing but rapidly decalcify. Use of hydrochloric acid should be limited to large resection specimens such as rib and the ability to be cut with a blade should be monitored every day.

The ctDNA is rapidly degraded by nucleases and contaminated by non-tumor DNA from white cells in whole blood [5]. Therefore, plasma should be separated as quickly as possible after the blood draw. Specialized cfDNA stabilizing tubes can be used for longer storage times. For future analysis, plasma should be frozen in single use fractions until DNA extraction [54].

WHICH PATIENTS SHOULD BE TESTED?

Molecular testing for targetable alterations should be performed in all patients with non-small cell lung cancer

It is uncontroversial that molecular testing for targetable alterations should be performed in all patients with adenocarcinoma or an adenocarcinoma component [6-8]. For tumors with other histologies, the CAP guideline recommends that biomarker testing may be performed when clinical features, such as young age and never-smoker, indicate a higher probability of targetable alterations [6]. The ASCO guideline recommends biomarker testing for patients with nonsquamous NSCLC or with squamous cell carcinoma who are light or never-smokers or younger than 50 years of age [7]. The NCCN guideline recommends biomarker testing for patients with large cell carcinoma or NSCLC not otherwise specified (category 1) [8]. For squamous cell carcinoma, tests for *EGFR* and *ALK* should be considered in never-smokers or small biopsy specimens, or those with mixed histology (category 2A) and tests for *ROS1*, *BRAF*, *MET*, and *RET* should be considered in small biopsies or mixed histologies (category 2A) [8].

In squamous cell carcinoma, the frequency of *EGFR* mutations is about 7%-8% and *ALK* fusion occurs in less than 1% [13,58,59]. The detection of *EGFR* mutations in small samples diagnosed as squamous cell carcinoma may be a result of incomplete sampling of adenosquamous cell carcinoma [59-62]. Because of histologic heterogeneity, small biopsies and cytology samples may not be representative of the total tumor and an adenocarcinoma component cannot be completely excluded. Undersampling of adenosquamous cell carcinoma is possible even in resection specimens of which only representative sections are submitted for pathologic examination. Several studies have

shown that the adenocarcinoma component and squamous cell carcinoma component of most adenosquamous cell carcinomas shared genetic alterations including *EGFR* mutations [60,63]. Restriction to adenocarcinoma histology may exclude some patients with squamous cell carcinoma from the potential benefits of targeted therapy. Therefore, molecular testing for targetable alterations should be performed in all NSCLC to select patients for targeted therapy.

HOW SHOULD THE RESULTS BE REPORTED?

Reporting should follow the quality control guidance of the Korean Society of Pathologists and the Korean Institute of Genetic Testing Evaluations

Reporting on molecular tests should follow the quality control guidance of the Korean Society of Pathologists (KSP) and the Korean Institute of Genetic Testing Evaluations (KIGTE). Reporting should provide information the clinician needs for arriving at treatment decisions. When reporting *EGFR* mutations, the clinical significance of the results should be included (e.g., response to EGFR TKI, resistance to EGFR TKI, or limited data on response). The sample adequacy and any limitations of the testing methods should be included for correct interpretation of the result by the clinician.

HOW SHOULD QUALITY CONTROL BE PERFORMED?

Internal and external quality control programs should be regularly implemented in accordance with the regulations of the Korean Society of Pathologists and the Korean Institute of Genetic Testing Evaluations

Regular quality control is necessary to maintain a high degree of reliability in molecular testing. The KSP and KIGTE provide guidance on internal quality control. Laboratories should also be enrolled in an external quality control and quality improvement program of the KSP and the KIGTE.

FUTURE PERSPECTIVES

With advances in precision medicine, NGS will become a routine practice. Tumor samples are often too limited to enable the detection of all targetable alterations with multiple single genetic tests. And biomarkers such as TMB can only be tested with NGS. Targeted NGS can rapidly and comprehensively detect multiple genetic alterations and save tumor tissue. The cost of

NGS is dropping rapidly. However, issues of reimbursement and approval with regard to NGS will need to be resolved for lung cancer patients to benefit from this technology. In USA, FoundationOne CDx, Oncomine Dx Target Test (Thermo Fisher Scientific, Carlsbad, CA, USA), FoundationOne Liquid CDx (Foundation Medicine), and Guardant360 CDx (Guardant Health, Inc., Redwood City, CA, USA) are currently FDA approved NGS panels as companion diagnostics for NSCLC patients. FoundationOne CDx detects genetic alterations in 324 genes and provides information on microsatellite instability and TMB using DNA isolated from FFPE tissue. Oncomine Dx Target Test detects genetic alterations in 23 genes using DNA and fusions in *ROS1* using RNA from FFPE tissue. FoundationOne Liquid CDx and Guardant360 CDx detects on genetic alterations in 311 genes and 55 genes, respectively, using ctDNA from plasma. And these two panels have been utilized for evaluation of blood TMB in clinical trials [19].

TMB may be used as a biomarker for immunotherapy in the foreseeable future. Although the FDA has approved high TMB as a biomarker for pembrolizumab treatment of solid tumors, TMB is still considered an emerging biomarker for NSCLC patients in the NCCN guideline [8]. Different assays, platforms and cutoffs to assess TMB have been used in many clinical trials and retrospective analyses [64]. Therefore, standardization of TMB assessment is key to its use in clinical practice. The ctDNA analysis could be used as a monitoring tool for treatment responses as recent clinical trials have demonstrated a correlation between changes in, or clearance of plasma EGFR mutational burden with EGFR TKI response [19,65]. However, consensus on how to appropriately measure treatment response has not yet been established.

CONCLUSION

In the era of targeted therapies and personalized medicine, the role of the pathologists is important in the management of NSCLC patients. At a minimum, tests for *EGFR*, *ALK*, *ROS1*, and *BRAF* should be performed for treatment decisions in all NSCLC patients. In the near future, *NTRK*, *MET*, *RET*, *HER2*, and *KRAS* tests will also be mandatory tests. Although advances in technology allow the detection of molecular alterations not only in tissue and cytology samples but also in ctDNA, tumor sample limitations are still a major challenge for molecular testing. Pathologists should pay attention to tumor saving strategies and enrichment through standardized and comprehensive approaches. Pathologists must also utilize a regular quality control program for

reliable analyses. New targeted therapies, predictive biomarkers, and technologies are continually emerging. KCPSG will keep pace with these advances through regular updates of these molecular guidelines for lung cancer patients.

Ethics Statement

Not applicable.

Availability of Data and Material

Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

Code Availability

Not applicable.

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Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

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