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Impact of Pre-Analytical Conditions on the Antigenicity of Lung Markers: ALK and MET

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Abstract: Diagnostic assays for molecular alterations highly correlated with prognosis, predictive efficacy or safety of therapeutics are valuable clinical tools and in some cases approved as companion diagnostics (CDx) by the Federal Food and Drug Administration. For example, assays that determine echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) translocation status have been approved as CDx assay for therapies that target this molecular alteration. Characterizing the parameters that may compromise diagnostic accuracy for molecular biomarkers is critical for optimal patient care. To investigate the impact of pre-analytical handling and processing of tumor tissue on commonly used diagnostic immunohistochemistry-based assays for ALK and mesenchymal epithelial transition protein [c-mesenchymal epithelial transition (c-MET)], we investigated the effects of cold ischemia, fixative type, fixation time, and cut-slide age on staining consistency and intensity using human lung xenograft tumor tissue. Cold ischemia times for up to 5 to 6 hours for c-MET or ALK, respectively had minimal impact on staining. The optimal fixation conditions for both assays were found to be at least 6 hours and up to 48 hours for c-MET or 72 hours for ALK, in 10% neutral buffered formalin and Zinc formalin. The ALK antigen demonstrated marked staining intensity differences across non-neutral buffered formalin fixative types and times. Finally, cut-slide age influenced assay performance for both ALK and c-MET, with maximum stability observed when cut slides were stored at ambient temperatures (30°C) for no longer than 3, and 5 months, respectively. This study highlights the potential for pre-analytical factors to confound diagnostic test result interpretation.

Key Words: pre-analytics, immunohistochemistry (IHC), anaplastic lymphoma kinase (ALK), c-mesenchymal epithelial transition (c-MET), non–small cell lung cancer (NSCLC)

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Lung cancer is among the leading causes of death in the United States and worldwide with an estimated 225,000 new cases and 155,870 deaths in 2017.[1] There are 2 main subtypes of lung cancer, small cell (SCLC) and non–small cell lung cancer (NSCLC). The most common type, NSCLC, represents ~85% of cases. Unfortunately, the majority of patients are diagnosed with late stage disease which is associated with poor prognosis and requires aggressive treatment which may include surgery, radiation, chemotherapy, and targeted therapy to maintain disease control. Accurate pathologic diagnosis is necessary to determine the optimal treatment options based on histological and molecular features.[2] Molecular testing guidelines for the selection of patients with lung cancer who may benefit from treatment with targeted tyrosine kinase inhibitors are available.[3]

One of the first diagnostic steps for NSCLC is to determine the histologic subtype using a panel of immunohistochemistry (IHC)-based assays in order to differentiate between squamous versus adenocarcinoma. This has become increasingly important because some treatment options, such as pemetrexed, are only indicated for patients with advanced nonsquamous cell NSCLC.[2] Additional molecular testing for alterations that might inform therapeutic decisions are now routinely performed for NSCLC cases.[3] For example, anaplastic lymphoma kinase (ALK) testing is routinely performed either by fluorescent in situ hybridization or by IHC. Detection of *ALK* gene rearrangements fluorescent in situ hybridization

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Animal Rights: All animals used in this study comply with Guide for the Care and Use of Laboratory Animals: Eighth Edition. Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council. ISBN: 0-309-15401-4, 248 pages, 6×9, (2010). This PDF is available from the National Academies Press at: www.nap.edu/catalog/12910.html

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was a standard for diagnosis and prescription of ALK-inhibitor therapy; however, IHC-based detection for ALK testing is rapidly gaining ground due to ease of use and assay improvements for enhanced sensitivity, reproducibility and antibody optimization.[4,5] The VENTANA anti-ALK (D5F3) CDx assay is FDA approved companion diagnostic (CDx) for the identification of patients with NSCLC who may benefit from treatment with the tyrosine kinase inhibitors (TKI's) that target ALK, including crizotinib, ceritinib, or alectinib.[4]

Both *ALK* gene rearrangements and *MET* gene amplifications have been observed to occur in lung adenocarcinomas and squamous cell carcinomas.[6] While the clinical benefit of targeting ALK with TKI's in patients who have ALK gene rearrangements has been clearly demonstrated, the clinical benefit of targeting c-mesenchymal epithelial transition (c-Met) has been less clear with mixed clinical benefit reported across different clinical studies in NSCLC investigating drugs that target c-MET. However, a recent meta-analysis that included 9 clinical studies involving c-MET inhibitors reported that c-MET therapies improved PFS in advanced or metastatic NSCLC patients.[5] In addition to being a promising independent therapeutic target, c-MET has been associated with resistance to epidermal growth factor receptor (EGFR) targeted therapies. On the basis of this observation, the combination of c-MET inhibitors with EGFR inhibitors have been investigated.[6] IHC assays for c-MET are available, but have not yet demonstrated prediction of clinical efficacy to c-MET directed therapies. Recent guidelines indicate c-MET molecular testing is not appropriate as routine stand-alone assay outside the context of a clinical trial. However, c-MET testing as part of a larger panel is considered appropriate when routine EGFR, ALK, BRAF, and ROS1 testing is negative.[3]

Pre-analytical handling and processing of tissue has potential to influence IHC assay performance; therefore, characterizing the impact of pre-analytical variables for each diagnostic assay is important and should guide the development of optimal tissue handling guidelines for that assay. There are multiple pre-analytic variables that can detrimentally affect IHC outcomes, including a delay to fixation (cold ischemia time), fixative type, and duration of fixation.[7–10] In order for diagnostic assays to be accurate, the biopsy (or resection) tissue samples taken from patients and the pre-analytical methods (such as fixation) used to preserve them for IHC testing should be consistent with pre-established criteria that have been validated for the IHC assay being used. Fixatives generally serve to halt cellular processes such as proteolysis, to preserve antigen morphology, as well as nucleic acids, and to protect the sample from microbial degradation.[11] This is achieved by sequestering proteins, for example, by cross-linking or denaturation. Prolonged ischemia (warm or cold) time before fixation may result in proteolytic degradation, thereby reducing antigenicity of the protein targeted by IHC.[8,10,12] Non-optimal duration of fixation may generate a variety of issues, ranging from partial fixation limited to the outer peripheries of the under-fixed tissues to diminished immunoreactivity as a result of over-fixation. Studies have observed that the type of fixative used influences the cross-linking within the tissue. Ideal fixatives balance maintenance of both morphologic features and antigen reactivity. A final, often-overlooked pre-analytic condition that can impact IHC performance is cut-slide age (or cut slide stability). Although chemical fixation is effective at preserving tissue in solid blocks, exposure of thin sections to the atmosphere (ie, oxygen and water) can lead to a decrease in staining, possibly via degradation of critical epitopes.[13]

This study reports on the impact of pre-analytical variables including cold ischemia time, fixation type, fixation time, and cut-slide stability on ALK and c-MET IHC assays using lung cancer xenograft tissues. The observations from these studies demonstrate that pre-analytical factors have the potential to impact diagnostic accuracy and interpretation for these assays and should be considered when establishing standard procedures for collection and processing of lung tumor tissue.

MATERIALS AND METHODS

Cell Lines

NCI-H2228 [human NSCLC adenocarcinoma (H2228); Cat # CRL-5935, ATCC, Manassas, VA] cells were grown in RPMI 1640 medium (Cat. # 30-2001, ATCC, Manassas, VA) supplemented with 10% Fetal Bovine Serum (FBS; Cat #16000-044, Thermo Fisher Scientific, USA) Penicillin/Streptomycin (Cat # 15140163, Thermo Fisher Scientific). Calu-3 (human metastatic lung adenocarcinoma; Cat # HTB-55, ATCC, Manassas, VA) cells were grown in IMDM (Cat # 12440, Thermo Fisher Scientific), supplemented with 10% FBS (Cat #16000-044, Thermo Fisher Scientific), nonessential amino acids (Cat# 11140-050; Thermo Fisher Scientific). Both cell lines were maintained in a humidified 5% CO_2 incubator at 37°C. Cells were routinely screened for mycoplasma and found to be negative and cell line authenticity was confirmed before they were implanted into severe combined immunodeficiency (SCID) mice.

NSCLC Samples

Commercially sourced human lung (NSCLC) patient resection specimens were procured from commercial vendors for use in research studies (US Biomax Inc. and ILSBio, LLC.).

Xenograft Samples

Xenograft Formation

Female SCID mice (CB17 Fox Chase SCID Mice; Charles River, Wilmington, MA) aged between 6 to 8 weeks were anesthetized by intraperitoneal injection of Ketamine/Xylazine/Sterile water (1:1:5) solution. H2228 cells or Calu-3 cells (1×10^7) suspended in 100 µl PBS were injected subcutaneously into the dorsal flanks. The animals were housed in micro-isolation cages and cared for according to guidelines for maintaining immunodeficiency models.

TABLE 1. Fixative Product Numbers and Source Used for ALK (D5F3) and c-MET (SP44) Fixation Studies

Fixative	Product Number and Source	H2228 (ALK)	Calu-3 (c-Met)
10% neutral buffered formalin	BDH0502-4LP BDH/Hydrol	Y	Y
Zinc formalin	Z2902 Sigma-Aldrich, St. Louis, MO	Y	Y
95% alcohol	7019-10 Macron Fine Chemicals	Y	—
Davidson's AFA	64133-14 Electron Microscopy Service	—	Y
B5	FXB5MPT American Master Tech	Y	—
Prefer	410 Anatech Ltd, Battle Creek, MI	Y	Y
Z-Fix	170 Anatech Ltd	—	Y
10% alcoholic formalin	ES784 Azer Scientific	Y	Y

ALK indicates anaplastic lymphoma kinase; c-MET, c-mesenchymal epithelial transition; Y, fixative tested; —, fixative not tested.

Xenograft Tumor Harvesting

Mice were sacrificed and Calu-3 tumor xenografts surgically excised when tumor volumes reached 1000 mm^3, typically 2 to 3 weeks postinjection. H2228 tumor xenografts were harvested at tumor volumes 250 to 900 mm^3, ~8 weeks postinjection. Tumors were dissected into 5 mm diameter cores and processed for fixation.

Xenograft Fixation Time and Fixation Type Studies

Excised tumors were immersed immediately in appropriate volumes of multiple fixatives (Table 1) for a range of time points. H2228 xenografts were fixed for 1, 6, 12, 24, and 72 hours, and Calu-3 tumors were fixed for 1, 3, 6, 12, 24, 48, and 72 hours.

Delay to Fixation (Cold Ischemia) Studies

To assess effects of delay to fixation, tumor pieces were fixed in 10% NBF for 24 hours following timed delays at room temperature (30°C). Timed delays from excision to fixation were 30 minutes, 1 hour, 2 hours, 6 hours, and 24 hours for H2228 tumors and 0, 1, 2, 3, 4, and 5 hours for Calu-3 tumors.

IHC

Specimens (lung tissue and/or xenograft) were embedded in paraffin and sectioned at 4 μm thickness for IHC. H2228 xenograft slides were stained using the VENTANA ALK (D5F3) CDx Assay, which is a rabbit monoclonal primary antibody (catalog #790-4796; Ventana Medical Systems, Tucson, AZ) on a fully automated VENTANA BenchMark XT instrument according to the manufacturer's specifications. For comparison, xenografts were also stained with CONFIRM anti-EGFR (B57) Rabbit Monoclonal Primary Antibody (catalog # 790-4347 EGFR antibody Ventana Medical Systems) and anti-Thyroid Transcription Factor-1 (SP141) Rabbit Monoclonal Primary antibody (catalog # 790-4756 TTF1 antibody, Ventana Medical Systems) on the BenchMark XT instrument. The *ultra*View DAB IHC Detection kit (Catalog # 760-500; Ventana) with reagent substitution [mouse HRP Multimer secondary antibody from the kit was replaced with OmniMap anti-Rabbit HRP-RUO (Catalogue # 760-4311; Ventana)] was used to complete the staining procedure. The reagent substitution eliminated background staining due to interaction with endogenous mouse Ig within the xenograft tissue. Calu-3 (ALK negative) xenograft slides served as the negative control.

Calu-3 xenograft slides were stained with CONFIRM anti-TOTAL c-MET (SP44) Rabbit Monoclonal Primary Antibody (catalog # 790-4430; Ventana Medical Systems) using VENTANA BenchMark ULTRA instrument according to manufacturer's specifications. The procedure was completed by using the *ultra*View Universal DAB Detection kit (Catalog # 760-500; Ventana) with reagent substitution (mouse HRP Multimer secondary antibody from the kit replaced by OmniMap anti-Rabbit HRP-RUO Catalog # 760-4311; Ventana) to limit background staining. The Calu-3 xenografts fixed for 12 hours in 10% NBF served as the reference standard (IHC intensity score: 2+).

IHC Scoring

A qualitative score criterion of 0 to 3+ with 0.25 point increments was used to evaluate IHC-stained slides. For ALK and c-MET studies, all slides were evaluated for the presence of IHC signal in majority of the cells by a qualified reader and scored using staining intensity criteria outlined in Table 2. A qualified reader is a reader that has been trained to read the assay for staining intensity criteria; however, is not a board-certified pathologist, and is not qualified to read specimens to provide a clinical diagnosis.

RESULTS

Differential ALK Staining in Clinical NSCLC Cases

When a large section of tissue is preserved in fixative, it is not uncommon to observe differential staining of

TABLE 2. ALK (D5F3) and c-Met (SP44) Qualitative Staining Intensity Criteria and Scores

Staining Intensity Score	ALK (D5F3) Staining Features for Scoring	c-MET (SP44) Staining Features for Scoring
0	Absence of specific signal	Absence of specific signal
1	Weak or punctate cytoplasmic staining	Membrane staining partial or circumferential, weak specific signal
2	Moderate punctate to granular cytoplasmic staining	Membrane staining typically circumferential, moderate specific signal
3	Strong granular cytoplasmic staining	Membrane staining thick and circumferential, strong specific signal

ALK indicates anaplastic lymphoma kinase; MET, mesenchymal epithelial transition.

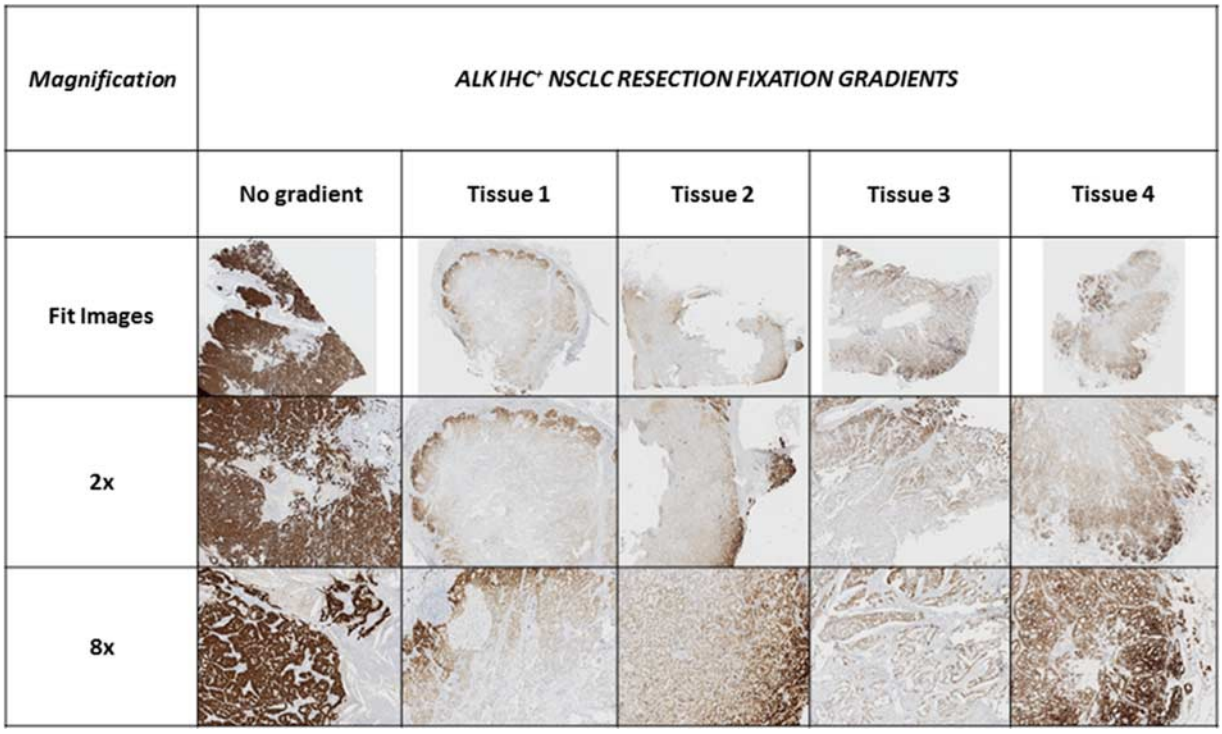

FIGURE 1. Fixation gradients potentially due to under-fixation in non–small cell lung cancer resection tissues stained with ALK (D5F3).

unstable epitopes between the edge or periphery versus the interior core of the tumor resection section due to fixative not adequately reaching the core. We observed this phenomenon in clinical NSCLC specimens stained for ALK (Fig. 1). The NSCLC resection samples display gradient staining intensities resulting in focal ALK staining. The likely reason for the fixation gradient observed in NSCLC resection tissues is under-fixation. This observation suggests that the pre-analytical collection and handling conditions of lung cancer specimens which will be stained by IHC for ALK should be optimized and standardized for optimal clinical interpretation.

Effect of Different Fixatives and Duration of Fixation on ALK and c-MET Staining

To determine the effects of fixative and fixation time, trends in staining intensity were assessed on IHC-stained xenograft specimens. Table 2 depicts the ALK (D5F3) and c-MET (SP44) qualitative staining intensity criteria and score methods used to evaluate all samples. It should be noted that a decrease in overall staining intensity in this study is not indicative of clinically positive or negative status as determination of the clinical status is validated on human tissue which is not appropriate for assessing xenografts. For example, for ALK IHC strong positive cells in each of the categories must remain present for cases to be designated as ALK-positive; however, for the purposes of this study, semiquantitative scores (between 1 and 3+) were used. Here a score of 2 signifies a decrease of staining expression as compared with a score of 3, while a score of 1 denotes a decrease of staining expression compared with the score of 2. It is ideal for the staining intensity to be within 0.5 points of the reference or gold standard.

Zinc-formalin and 10% NBF-fixed H2228 xenograft tumors demonstrated optimal ALK (D5F3) staining (score: 2.5) after 6 hours of fixation. Figure 2 shows the effect of 10% NBF fixation for 1 hour (minimal staining, score: 1+), 12 hours (optimal staining, score: 3) and remaining within 0.5 of the 3 score through 72 hours. Times >24 hours exhibited staining intensities qualitatively similar to the 6 and 12 hours times, indicating that a longer fixation time is not detrimental to staining intensity for ALK but "under-fixation" (<6 h) impacts ALK detection. Treatment with several other fixatives (B5, Prefer, AFA, and 95% alcohol) resulted in severely compromised ALK (D5F3) staining intensity at all-time points ranging from 1 to 72 hours, with scores ranging from 1+ to 2+ (AFA); 0.5 to 1+ (95% alcohol); 0.75 to 1.75 (Prefer); 1.0 to 1.75 (B5 fixative). In the case of ALK detection, the performance of zinc formalin fixation for at least 6 hours was comparable with 10% NBF. TTF1 and EGFR, which were used as comparator biomarkers expressed in lung cancer, were unaffected by fixation type and duration of fixation (Supplemental Material, Supplemental Digital Content 1, http://links.lww.com/AIMM/A219; Supplemental Digital Contents 2 and 3, Supplemental Fig. 1, http://links.lww.com/AIMM/A220; Supplemental Fig. 2, http://links.lww.com/AIMM/A221). These data indicate that the ALK staining intensity by IHC

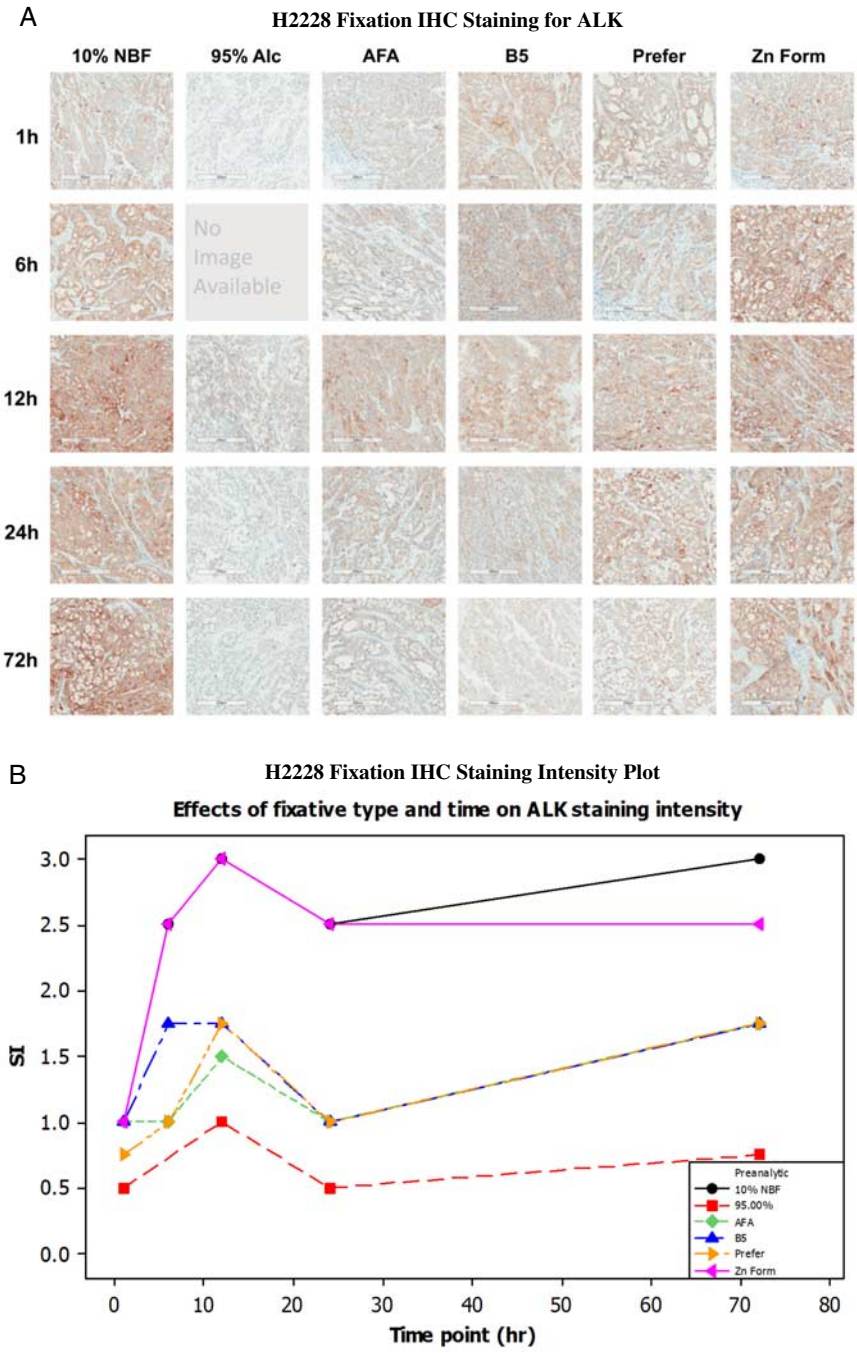

FIGURE 2. H2228 Fixation IHC staining images and staining intensity for ALK (D5F3). A, Representative images (×20 magnification) of H2228 lung cancer xenograft tumor tissue stained for ALK (D5F3) following the indicated times of fixation in indicated fixatives. B, Graphical illustration of the impact of fixation time and fixative on staining intensity.

is impacted to a much greater extent by fixation conditions compared with other lung biomarkers (EGFR and TTF1).

Effect of Different Fixatives and Duration of Fixation on c-MET

For the assessment of c-Met staining, Zinc formalin and Z-Fix fixation both yielded robust staining of Calu-3 xenografts. Alcohol-based fixatives Alcohol Formalin and AFA presented lower staining intensities. Fixation in 10% NBF showed steady enhancement from 1 through 6 hours, followed by equivalent staining (score: 2+) from 6 to 48 hours before declining slightly at 72 hours (Fig. 3). At the 6 hours time point, 10% NBF, Zinc formalin, B5 and Prefer demonstrated optimal staining (score: 2+), which remained consistent at 12 and 48 hours time points and

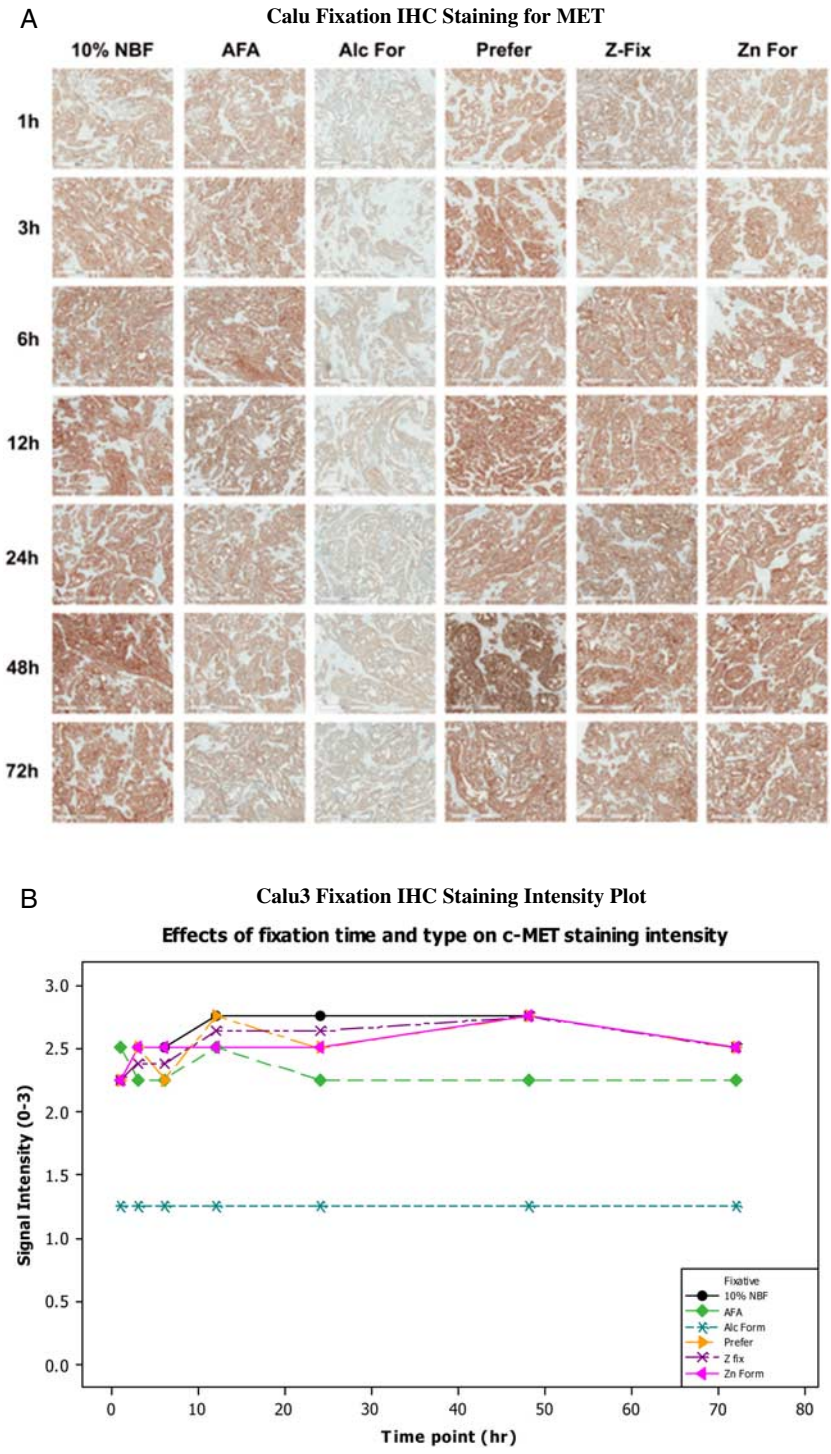

FIGURE 3. Calu-3 fixation IHC staining images and staining intensity for c-MET (SP44). A, Representative images (×20 magnification) of Calu-3 lung cancer xenograft tumor tissue stained for c-MET (SP44) following the indicated times of fixation in indicated fixatives. B, Graphical illustration of the impact of fixation time and fixative on staining intensity.

dipped at 72 hours (Fig. 3). Alcoholic formalin fixation yielded suboptimal staining throughout the time course (Fig. 3), with a drop of staining intensity from baseline score of 2+ to 1+. Together, these results support that pre-analytical conditions related to fixation type and time may impact measurement of c-MET by IHC, with alcoholic fixatives having the potential to cause the most detrimental impact clinical interpretation.

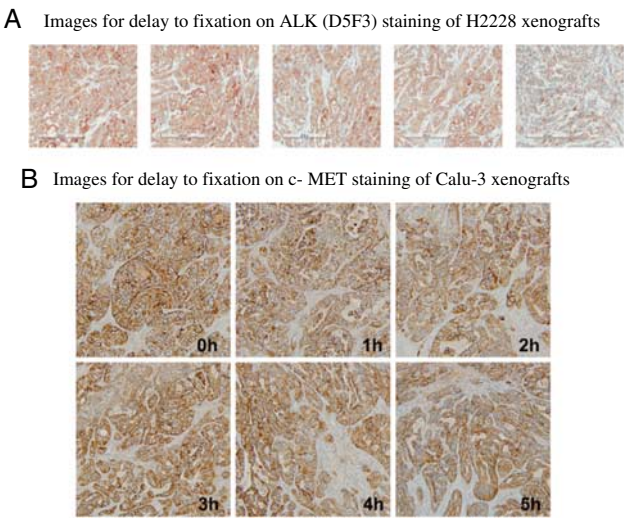

FIGURE 4. A, Representative images (×20 magnification) of ALK (D5F3) stained of H2228 xenografts following delay in fixation. H228 xenograft tumor tissue was kept under cold ischemia for the times indicated before fixation in 10% neutral buffered formalin. B, Representative images (×20 magnification) of c-MET (SP44) stained of Calu-3 xenografts following delay in fixation. Calu-3 xenograft tumor tissue was kept under cold ischemia for the times indicated before fixation in 10% neutral buffered formalin.

Effects of Delay to Fixation (Ischemia) on ALK and c-MET Staining

For delay to fixation (also known as ischemia) 30 minutes, 1 hour, 2 hours, 6 hours, and 24 hours time delays after harvesting and before fixation in 10% NBF were selected for each xenograft. The staining intensities were compared with the xenografts immediately fixed in 10% NBF. For ALK (D5F3), there was little-to-no difference in ischemic samples staining intensity for the time points of 30 minute, 1 hour, 2 hour, and 6 hours. Each of these time points was scored as a 3 for staining intensity. Delay to fixation time (ischemia) of >6 hour decreased ALK staining intensity in 10% NBF fixed H2228 xenografts (Fig. 4a), while TTF1 and EGFR staining intensity remained robust and unaffected by fixation delay of up to 24 hours (Supplemental Material, Supplemental Digital Content 1, http://links.lww.com/AIMM/A219). The delay to fixation studies in c-MET in Calu-3 xenograft tissue indicate consistent staining intensities for up to 5 hours after tumor excision from the animal (Fig. 4b). However, best clinical practice should still be immediate placement of tissues in fixative, and ASCO/CAP guidelines[14,15] recommend <1 hour delay to fixation.

Epitope Shelf Life and Stability

Stability assessment of the ALK and MET epitopes suggest that cut slides of NSCLC can be stored at ambient temperatures (30°C) for no longer than 3 months, and 5 months, respectively (Supplemental Material, Supplemental Digital Content 1, http://links.lww.com/AIMM/A219; Supplemental Digital Contents 4 and 5, Supplemental Table 1, http://links.lww.com/AIMM/A222; Table 2, http://links.lww.com/AIMM/A223).

DISCUSSION

Fixative type, time, and delay to fixation strongly influenced ALK IHC detection in tumor xenografts. For both ALK and c-MET, fixation time of 6 hours or greater was optimal and extending the fixation time had little impact on staining intensities, while the type of fixative used showed drastic differences. Overall, alcohol-based fixatives (AFA, Alcoholic Formalin) demonstrated poor performance in both ALK and c-MET. Glyoxal-based fixative (Prefer) showed inconsistent staining intensities across all time points for c-MET, whereas 10% NBF, Zinc formalin and Z-Fix fixation for 6 hours and beyond emerged as the best fixative types among all fixatives tested. Fixation time <6 hours indicated suboptimal IHC staining signal intensities for all cases tested. On the basis of our results and prior feasibility studies, alcohol-based and glyoxal-based fixatives are not recommended for optimal detection of c-MET or ALK. The range of variation across different fixative types and times show declining or suboptimal staining in alcohol and glyoxal-based fixatives. This indicates the need for identification and adherence to a standard fixative and protocol that works satisfactorily and consistently when staining for ALK or c-MET. Overall, the results of the current study suggest that the tissue fixation guidelines provided by ASCO/CAP for estrogen receptor, progesterone receptor, and epidermal growth factor receptor 2 testing which includes fixation of breast tumor tissue in 10% NBF for at least 6 hours and up to 72 hours is also appropriate for lung tumor tissue staining by IHC for ALK and c-MET.[14,15]

Cut-slide age influenced assay performance for both ALK and c-MET, with maximum stability observed when cut slides were stored at ambient temperatures (30°C) for no longer than 3, and 5 months, respectively (Supplemental Material, Supplemental Digital Content 1, http://links.lww.com/AIMM/A219). This study highlights the potential for pre-analytical factors to confound diagnostic test result interpretation.

The ASCO/CAP guideline[14,15] for pre-analytic variables for lung cancer biomarker testing indicates that, "Pathologists should use formalin-fixed, paraffin embedded specimens or fresh, frozen, or alcohol-fixed specimens for PCR-based mutation tests. Other tissue treatments, eg, acidic or heavy metal fixatives, or acid decalcifying solutions) should be avoided in specimens destined for EGFR testing." These guidelines are currently under review and revision to encompass generalized molecular markers instead of referencing solely EGFR. Without formal standardization of the fixation process for utilization with IHC, many laboratories have developed and use their own processes for fixation.[7] The process by which fixatives are selected and used for tissue

fixation are defined most often locally by each laboratory and then validated appropriately for use.[16] A lack of standardization leads to unknown effects on the quality of the IHC staining and associated clinical interpretation of the assay. Although it is clear that all antigens do not require specific fixation variables to be detected; the tissue used for NSCLC IHC panels will only be fixed once; and therefore, tissue should be fixed in a manner that will preserve the most sensitive of the antigens.

Although the results within this study are compelling with regard to the importance of pre-analytical variables on the assays investigated, the findings must be viewed in light of the experimental design limitations. The use of xenograft tissue rather than patient lung cancer samples was a practical limitation driven by the difficulty of obtaining fresh tumor tissue in the time frame and quantity needed to include the many parameters investigated. In addition, fine-needle aspirates, core needle biopsies, or cytology samples, are not represented or addressed directly in the current study design. Further investigation into the impact of pre-analytics would be beneficial for these sample types.

Together, these data indicate that the c-MET and ALK antigen require proper pre-analytical conditions to give reliable IHC results. This work supports further efforts to broadly develop, validate, and adopt appropriate guidelines and standard procedures to optimize NSCLC fixed tissue quality in order to ensure diagnostic accuracy of IHC-based assays.

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