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Immunocytochemistry for Predictive Biomarker Testing in Lung Cancer Cytology

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Abstract

With an escalating number of predictive biomarkers emerging in non–small cell lung carcinoma (NSCLC), immunohistochemistry (IHC) is being used as a rapid and cost-effective tool for the screening and detection of many of these markers. In particular, robust IHC assays performed on formalin-fixed, paraffin-embedded (FFPE) tumor tissue are widely used as surrogate markers for

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CONFLICT OF INTEREST DISCLOSURES

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ALK and *ROS1* rearrangements and for detecting programmed death ligand 1 (PD-L1) expression in patients with advanced NSCLC; in addition, they have become essential for treatment decisions. Cytology samples represent the only source of tumor in a significant proportion of patients with inoperable NSCLC, and there is increasing demand for predictive biomarker testing on them. However, the wide variation in the types of cytology samples and their preparatory methods, the use of alcohol-based fixatives that interfere with immunochemistry results, the difficulty in procurement of cytology-specific controls, and the uncertainty regarding test validity have resulted in underutilization of cytology material for predictive immunocytochemistry (ICC), and most cytopathologists limit such testing to FFPE cell blocks (CBs). The purpose of this review is to: 1) analyze various preanalytical, analytical, and postanalytical factors influencing ICC results; 2) discuss measures for validation of ICC protocols; and 3) summarize published data on predictive ICC for *ALK*, *ROS1*, *EGFR* gene alterations and PD-L1 expression on lung cancer cytology. Based on our experience and from a review of the literature, we conclude that cytology specimens are in principal suitable for predictive ICC, but proper optimization and rigorous quality control for high-quality staining are essential, particularly for non-CB preparations.

Keywords

cell blocks; immunocytochemistry; lung cancer; predictive; smears

INTRODUCTION

The treatment of advanced non-small cell lung carcinoma (NSCLC) has undergone a paradigm shift in recent years.[1] Targetable alterations in epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), and ROS proto-oncogene 1(*ROS1*) genes are present in approximately 20% of NSCLCs in western populations, and in view of their excellent response to oral *EGFR/ALK/ROS1* tyrosine kinase inhibitors, the latter have replaced conventional platinum-based chemotherapy as a first-line therapy for patients with the corresponding genetic alterations.[1] Accordingly, current guidelines mandate testing for these alterations in all patients with advanced nonsquamous NSCLC.[2]

The discovery of a subset of NSCLCs responding to immune-checkpoint inhibition by programmed death 1/programmed death ligand 1 (PD-1/PD-L1) monoclonal antibodies has opened new therapeutic avenues in *EGFR/ALK/ROS1* wild-type advanced NSCLC, including squamous cell carcinoma.[3] Nearly 30% of these patients may be eligible for a first-line immune-checkpoint inhibitor based on high PD-L1 expression levels by immunohistochemistry (IHC).[4] Aptly, PD-L1 testing has become the standard of care for all advanced NSCLC.[1]

With an escalating number of predictive biomarkers emerging in NSCLC, IHC has been used as a rapid, cost-effective alternative to fluorescence in situ hybridization (FISH) and molecular testing in the screening of several of these alterations (Table 1). For validated predictive IHC assays, specific thresholds for positivity, guidelines for validation of laboratory-developed tests, controls, and the influence of various analytical factors have been studied mainly on formalin-fixed, paraffin-embedded (FFPE) histology samples.[5] With

advances in minimally invasive diagnostic procedures that yield predominantly cytology samples, there is increasing demand for predictive biomarker testing on cytology samples as well.

Assay revalidation is required when a validated IHC assay is performed on cytology specimens due to differences in their processing techniques.[6] In general, cytology samples show greater variability in preanalytical factors, including sample types, procurement, storage conditions, preservative media, fixatives, processing techniques, and stains, with resultant difficulties in standardization of immunocytochemistry (ICC). FFPE cell blocks (CBs) are easier to incorporate into existing IHC protocols, thus constituting the predominant type of cytology preparation that has been used for biomarker testing in most studies.[7] Endobronchial ultrasound-guided transbronchial needle aspirate (EBUS-TBNA) samples can be formalin-fixed cytology specimens, though this depends on whether they are treated in the laboratory as histology or cytology specimens. If collected in formalin, CBs show high concordance in staining with matched surgical specimens for most routinely used IHC markers,[8] and show significantly superior quality of staining on average.[9] However, they are time-consuming, costlier, more technically challenging, not uniformly available across all laboratories, and frequently lack adequate cellularity.[7,10,11] Some of newer improvised CB methods such as Cellient and Cell-Gel claim to yield better cellularity[7]; however, they use nonformalin fixatives and therefore still require revalidation.[6] Using only CBs for ICC may result in a large fraction of non-CB preparations remaining unused for ICC, which may subject patients to unwarranted repeat sampling.

Although approximately 51% and 75% of cytology laboratories in the United States[12] and Europe,[13] respectively, reported using non-CB preparations for diagnostic ICC in surveys from 6 to 7 years ago, their use for ICC has likely declined more recently—particularly in the United States, where commercial laboratories do not accept smears for ICC. There is a paucity of articles outlining best practice guidelines for ICC,[14–20] and many perform diagnostic ICC without adequate validation or quality control measures.[9,12,13,21] In an external quality assessment program conducted in Europe, a high quality of immunostaining, regardless of methods or fixatives, was observed on a variety of cytology preparations received from different laboratories,[9] and there is good evidence that with appropriate modifications in analytical factors, ICC on non-CB preparations can be equivalent to ICC on FFPE CB sections.[22–26]

In a recent article, key questions of diagnostic IHC in lung cancer have been addressed, and practice recommendations have been made both for histological and cytological specimens.[27] In this review, we discuss the role of preanalytical, analytical, and postanalytical factors that influence the success of ICC testing in various cytology preparations, suggest measures for validation and quality control, and summarize the currently available data on predictive ICC in lung cancer cytology specimens. This paper represents an official statement of the IASLC Pathology committee.

IMMUNOCYTOCHEMISTRY

ICC refers to the application of immunochemistry to cytological material. The most frequent cytology specimens that undergo ICC testing are aspirates derived from lung, breast, thyroid or deep organs, serous fluids, and urine.[13] ICC has been attempted on virtually every type of cytology specimen and preparation,[9,12,15,16] and the following factors appear to influence ICC results to varying degrees.

Preanalytical Factors

Fixatives—Alcohol-based fixatives are used in cytology due to its rapid action, ability to clear mucous and obscuring blood, and an overall improved morphology. The most widely used fixatives in non-CB cytology include the ethanol-based Saccomanno (50% ethanol and 2% polyethylene glycol), Delaunay (equal parts ethanol and acetone admixed with 0.5 mL of 1M trichloroacetic acid), and commercial spray fixatives, while the methanol-based CytoLyt or PreservCyt solutions (the latter being optimized for the ThinPrep liquid-based cytology slide preparation system) (Hologic, Marlborough, Massachusetts) serve as preservatives and transport media. The ethanol-based hemolytic CytoRich Red collection fluid (Thermo Fisher Scientific, Waltham, Massachusetts) also contains formalin. All of these fixatives and transport media can be problematic for ICC, and frequent discordant results with IHC have been obtained for some antibodies.[16,22,28–33] In a comparative study of estrogen receptor, progesterone receptor, and Her2 ICC between formalin- and Saccomanno-fixed preparations, the latter showed more variable staining and increasing false-negative results, with longer durations of fixation.[33] Methanol-based fixatives such as CytoLyt and PreservCyt (a fixative for the Cellient automated cell block system [Hologic]) also show decreased staining intensity and frequent false-negative results.[22,28–33] Although some early studies noted frequent false-negative staining only for nuclear antigens,[16,32] later studies observed such discrepancies with cytoplasmic and membranous antigens as well.[22,30,31] Nevertheless, in the United Kingdom National External Quality Assessment Service (UK NEQAS) for ICC, with the exception of acetone that showed inferior ICC quality due to poor morphological preservation, nearly all nonformalin fixatives—including Delaunay-, methanol-, and ethanol-based solutions and the ethanol- and formalin-based CytoRich Red—yielded a quality of ICC staining similar to that of formalin alone.[9] Thus, a laboratory may use any of these fixatives as suited to their needs, the only caveat being the need to revalidate their ICC procedure before clinical application.

Preparations—With the exception of filter preparations, most cytology preparations[34–47] are feasible for ICC, each with its own advantages and disadvantages (Table 2). Among non-CB preparations, alcohol-fixed, Papanicolaou direct smears (DSs) and cytospin preparations (CSs) have been most commonly used for ICC, with excellent results,12,17,25,34–36,38–41 and are the standard media for ICC in many laboratories.[42–47] Papanicolaou or unstained liquid-based cytology (LBC) preparations have also been used for ICC with comparable results.[37,46] Variable results have been obtained with air-dried DSs and CSs, with some authors reporting complete lack of staining,[12,34,38,40] whereas others report successful ICC on unstained slides postfixed in formalin[48] and/or alcohol/methanol-acetone[16,41,49] and on

Diff-Quik–stained smears postfixed in formalin.[50] Saline-rehydrated air-dried smears fixed in 95% ethanol + 5% acetic acid are unsatisfactory for ICC.[41]

Stains—Papanicolaou or Diff-Quik stains do not appear to interfere with ICC results, and a separate destaining procedure merely contributes to unnecessary cell loss and damage.[38,40,50] The Papanicolaou counterstains EA50 and OG6 are usually bleached out during endogenous peroxidase blocking and/or during antigen retrieval.[17]

Coverslipping and mounting media—Prior to ICC, the prestained slides of non-CB cytology need to be soaked in xylene to dissolve the nonaqueous permanent mounting media and remove the coverslip. This process takes a few hours for cases of fresh specimens from daily practice but may require up to several days in retrospective studies, depending on archival time. Notably, the epitopes in previously stained alcohol-fixed cytology slides have been shown to remain intact during an archiving period of at least 1 to 2 years if properly sealed by a coverslip.[51] In contrast, it has been shown that epitope stability on empty sections of FFPE blocks is compromised as early as 3 to 6 months at room temperature, depending on the epitope.[52] The same limitation might apply to non-coverslipped smears, although this has yet to be analyzed systematically.

Analytical Factors

Antigen retrieval—In contrast to formalin, which is a cross-linking fixative that masks most epitopes, alcohol-based fixatives work by causing tissue dehydration and protein coagulation. Thus, the majority of cytoplasmic and membranous antigens do not require any form of antigen retrieval for ICC.[17,32,39] In a study of 43 commonly used antibodies, Denda et al[17] found that heat-induced antigen retrieval (HIAR) was essential for uncovering epitope reactivity for all nuclear antigens and a subset of cytoplasmic and membranous antigens in alcohol-fixed Papanicolaou-stained smears. The heat likely plays a role in overcoming steric hindrances and allowing access of antibody to epitope binding sites. Antigen retrieval buffer solutions of different pH and chemical compositions did not alter results significantly and citrate buffer at pH 6.0 was suitable for most antibodies. HIAR has also been found to improve ICC staining for certain antigens on ThinPrep LBC preparations[37] and for air-dried smears postfixed in formalin.[48] A shorter duration of HIAR is usually sufficient for cytology smears compared with FFPE tissue. Long durations can cause damage and floating of tissue sections,[53] and HIAR should be optimized separately for each antibody.

Protease antigen retrieval should be avoided for non–formalin-fixed cytology preparations due to excessive protein digestion leading to epitope loss and increased background staining.[15,17,54]

Other factors—High antibody titres can cause nonspecific staining, and antibody dilutions must be customized for ICC.[13,54] Peroxidase blocking in inflammatory smears should be done to prevent false-positive staining.[54] ICC results also depend on the sensitivity of the antibody detection method (eg, polymer refine kit, OptiView, UltraView, tyramide amplification) used.[22] Use of automated staining platforms would improve the standardization and reproducibility of ICC results, but published data on non-CB

preparations are still scarce.[15] Some of the authors (D.J., A.N., S.S.P., L.B.) worked with the Leica Bond autostainer[51,55] and the Ventana BenchMark XT,[44,46] while the Ventana BenchMark Ultra[56] and the Dako autostainers[57,58] have been used by others. If using manual staining, the number of cytotechnologists involved should be as small as possible to reduce operator-induced variations in procedure.[16]

Postanalytical Interpretation

Crushed and degenerated cells or presence of marked necrosis can cause nonspecific staining, and these areas should be avoided for ICC interpretation.[15,54] Large 3-dimensional cell clusters entrap reagents and can show false-positive staining in the center of the cell groups.[15,55] Interpretation should be made on isolated tumor cells or in distinct 2-dimensional clusters. Cells in exudative effusions may nonspecifically adsorb antigens present in the fluid, resulting in false-positive membranous staining. Cytocentrifuged smears from such fluids may also develop a layer of precipitated proteins over cellular material during fixation, obscuring antibody penetration and resulting in false-negative ICC with increased background staining. Both of these phenomena can be eliminated by prewashing with an isotonic saline solution.[15] Other sources of false-positive staining include nonspecific uptake by histiocytes, macrophages, and giant cells.[15,55,59,60]

In smears prepared from tumors with fragile cytoplasm such as high-grade lymphomas, the cell damage caused by smearing can cause high background staining, and appreciation of true membranous and cytoplasmic staining may be difficult.[16] Similarly, cytoplasmic staining may be easily missed in cells with scant cytoplasm, as in signet ring cells. Nuclear staining is generally easier to interpret than cytoplasmic staining in cytology smears.

Analytical Validation, Controls, and Quality Assessment

Analytical validation of an IHC assay requires an appropriate number of control samples (equal numbers of known positive and negative controls). Notably, these samples must be “processed the same way” as the patient samples that are to be tested using the assay under validation.[6] Nearly two-thirds of cytology laboratories in Europe and United States report usage of FFPE histology sections as controls for ICC in two independent surveys,[12,13] while a meta-analysis of 100 published ICC-based cytology articles found that only 13% of them even specify the use of cytology-specific controls.[21] FFPE histology controls are at best acceptable if ICC is performed on FFPE CBs but are not adequate for non–formalin-fixed preparations.[6] Use of commercially available in vitro cell lines with known levels of target antigen expression may circumvent this difficulty and are available for *ALK*, *ROS1*, *EGFR*-mutant and PD-L1 IHC assays (Supporting Table 1). These preparations can be maintained in culture indefinitely, processed into CSs or CBs in the same way as the cytology sample being tested, and control slides can be coverslipped and/or refrigerated to preserve the epitopes.[61] However, such cell lines are costly and not available for all antigens. Alternatively, we recommend processing leftover effusion fluids or brushings from cut surfaces of unfixed fresh organs (either normal or cancerous) into controls and stored as cytospin smears or LBC slides.[14,50]

After establishing and optimizing the analytical protocol using these positive controls, there is a need for further validation. This is particularly challenging in cases of rare *ALK*- or *ROS1*-positive NSCLC in non-CB cytology. To maximize the number of cases for validation, it is advisable to use archived surplus slides from known positive and negative cases. A predictive ICC assay can also be validated by comparison with standard molecular tests[11,51,55–57,60,62–70] or with clinical response.[71] To improve and expand initial validation on retrospective material, it can be continued prospectively in parallel with a validated comparator used in clinical practice during a period of time (eg, FISH in case of ALK and ROS1 or a validated IHC assay on a matched histological specimen).

In a College of American Pathologists survey of 1899 cytology laboratories, only 4 out of 345 cytology laboratories that performed ICC even attempted to validate predictive Her2neu ICC on non-CB preparations.[12] While 3 of these laboratories successfully validated alcohol-fixed smears, 1 failed to validate air-dried smears for ICC.[12] Thus, while it is difficult to validate ICC for every type of cytology preparation and antibody, use of the consortium approach as offered by NordiQC and UK NEQAS, wherein sharing of validated protocols is performed among different laboratories will help in development of standardized ICC assays.[12] In addition, irrespective of the method of validation, routine internal quality control checks and participation in external quality control programs will further improve ICC practices.[9] Monitoring the results of local immunochemistry biomarker testing for comparison with data from the literature and other laboratories is another attractive tool for continuous quality control, as is currently being used in Switzerland at the Biopath platform for PD-L1 testing in routine diagnostic practice.[72]

PREDICTIVE ICC IN LUNG CANCER CYTOLOGY

ICC for ALK Rearrangements in NSCLC

ALK immunohistochemistry using D5F3 clones shows high sensitivity and specificity for *ALK* rearrangements (*ALK*-R) and is now approved for patient selection for ALK TKI.[2,5] The tyramide amplification system in the D5F3 Ventana automated assay results in enhanced positive staining and uses a bimodal staining interpretation (positive vs negative) that is less subject to interobserver variability. The 5A4 clone on laboratory-developed tests shows a more dynamic range of staining intensity (0 to 3+) with varying proportions of those with 1+ and 2+ intensities lacking *ALK*-R on FISH. However, when used on an automated platform, such equivocal results are reduced, and it shows comparable performance with D5F3 Ventana assay.[73]

Preparations—The majority of studies on ALK ICC have been performed on FFPE CBs without modification of the IHC procedure, using 5A4 or D5F3 clones, on various automated staining platforms.[55,62–68] These studies have uniformly demonstrated 100% sensitivities, albeit with variable specificities (83%−100%), for the presence of *ALK*-R by FISH. One study found that ICC was more sensitive (100%) than reverse transcription–polymerase chain reaction (88%) or FISH (62%) for the detection of *ALK*-R on CB sections.[68] The lower sensitivity of FISH in this study was attributed to the insufficient number of tumor cells (<50), which led to a higher number of inconclusive FISH results,

unlike ICC, which is interpretable even in few tumor cells.[66,68] Alcohol-fixed DSs,[55,58,59,69,74] air-dried DSs,[69] CSs,[55] and LBC preparations[55,69] have also been evaluated for ALK ICC. With the exception of 2 studies that reported 100% sensitivity on alcohol-fixed smears,[55,75] other studies have reported relatively poor sensitivities for *ALK*-R detection by ICC, ranging from 66% to 86%.[56,57,69,74] Notably, none of the latter studies used the highly sensitive Ventana D5F3 assay that has been used in most CB studies, and they all used different scoring criteria for positivity thresholds (Supporting Table 2).

Fixatives—Non-formalin fixatives, including PreserveCyt,[11] have not been found to interfere with ALK ICC results.[69] Savic et al[55] performed ALK ICC using 5A4 clone on paired FFPE CBs and Delauney-fixed Papanicolaou-stained smears with 100% concordance. ICC-specific positive controls in the form of alcohol-fixed CSs (for ICC on smears) and FFPE CB sections (for ICC on CBs) from the H3122 *ALK*-R cell line were used for validation of the procedure. ICC was optimized by changes in antibody dilution, duration of pretreatment antigen retrieval, and duration of primary antibody incubation.

Minimum number of tumor cells required for ALK ICC—Zhang et al[57] specified a cutoff of 200 tumor cells for successful ICC testing on Papanicolaou-stained smears. However, most studies report that ALK staining is generally diffusely positive irrespective of the percentage of tumor cells showing *ALK*-R signals on FISH and samples with even scant tumor cells may be valid for ICC interpretation (Fig. 1B).[66,68]

Staining platforms for ICC—Most studies have used automated immunostainers, and manual ALK ICC has been evaluated in only 1 study.[74] Although this study is limited by the small number of cases and lack of FISH confirmation, ICC showed 94% (17/18) concordance with IHC results. Citrate acid at pH 6.0 was used for antigen retrieval, with the duration of the latter being shorter than that for IHC.

ICC for ROS1 rearrangements in NSCLC

ROS1 IHC using the D4D6 clone is highly sensitive, but it is relatively less specific and has been recommended only as a screening tool, and it requires confirmation by an alternate method before treatment selection.[2] Unlike ALK, specific cutoffs have not been established, even on IHC, and there is poor understanding about the clinical significance of ROS1 IHC+ cases lacking *ROS1* rearrangements on FISH.[76] Full-length ROS1 protein can be expressed in normal cells and weak expression of ROS1 has been noted in bronchial epithelial and basal cells, peribronchial glands, and smooth muscle cells, hyperplastic type 2 pneumocytes, metaplastic bronchiolar cells, and alveolar epithelial cells.[59] Furthermore, ROS1 expression is more dynamic than ALK expression, and positivity has been found to be of variable intensity within *ROS1*-rearranged cancers and cell lines,[51,59] resulting in difficulties in determining ideal cutoffs for positivity. Nevertheless, the occurrence of heterogeneous staining patterns, (ie, completely negative and strongly positive areas within the same tumor, as seen in PD-L1 staining) is uncommon in *ROS1* rearranged tumors.[77] Most authors have suggested H-scores of 100–150 or a ≥2+ intensity of cytoplasmic staining in 50% to 75% of tumor cells to yield satisfactory sensitivities and specificities.[60,77–79] ROS1 IHC has been recommended to be scored only in specimens with ≥20 tumor cells,[79] whereas 50 tumor

cells are required for FISH testing. However, a positive result even in fewer neoplastic cells may be considered diagnostic,[51,59] and the specimen should be processed for confirmatory FISH testing.

FFPE CBs[60,77] and alcohol-fixed, Papanicolaou-stained DSs and CS[59] have all been reported to be suitable for ROS1 ICC (Supporting Table 3, Fig. 2). In the only systematic analysis of the sensitivity and specificity of ICC for *ROS1* rearrangements, Vlajnic et al[51] tested 295 alcohol-fixed, Papanicolaou-stained smears, including archived smears up to 4 years old, for ROS1 ICC. Diffuse staining, albeit with varying intensity across tumor cells of a given case, was seen in 13 cases, all of which were confirmed to harbor *ROS1* fusions on FISH or next-generation sequencing. No specific cutoff was used, and any intensity of cytoplasmic staining was interpreted as positive.

A new Ventana ROS1 (SP384) antibody has been developed recently that showed a high positive (100%) and negative (90.5%) percent agreement when using a standardized protocol on Ventana Benchmark Ultra at a cutoff of ≥2+ staining in cytoplasm in >30% of the total tumor.[80] However, data on cytological specimens are not yet available.

ICC for EGFR Mutations in NSCLC

Antibodies recognizing the most common mutated forms of the EGFR protein—namely, the L858R substitution on exon 21 (43B2) and the E746-A750 15-bp deletion on exon 19 (6B6) of *EGFR*—are available.[2] These antibodies, although highly specific (96%−99%), do not recognize other less common types of EGFR mutant proteins, including those resulting from variant exon 19 deletions, and may show false-positive staining with treatment-insensitive exon 20 insertions.[81] While previous guidelines allowed their use by IHC in settings with very limited material, the predominantly low sensitivity reported in various studies (~47% −92%)[82] has led to the current guidelines no longer recommending these antibodies for patient selection.[2] Despite these limitations, IHC assays using these antibodies do show high specificity and positive predictive value for their respective mutant proteins and may be of value for rapid screening in paucicellular cytology preparations that lack sufficient material for molecular testing.[19] Understandably, negative IHC/ICC results do not exclude the presence of EGFR mutations and require molecular analysis.[19]

A variety of cytology preparations including alcohol-fixed DSs,[82–84] CSs,[82,83] LBC preparations,[85,86] and CBs have been found suitable for EGFR mutant-specific ICC, while air-dried preparations show suboptimal antigen preservation.[83] Some authors have used both manual[85,87] and automated platforms for ICC, and 1 study also evaluated novel antibody clones (SP111 and SP125 from Ventana) on cytology samples with similar results[84] (Supporting Table 4).

Limitations

On FFPE samples, moderate intensity 2+ membranous staining in >10% tumor cells is usually considered positive.[19] However, cytology specimens show decreased intensity of staining compared with matched FFPE tissue, thus leading to reduced sensitivity.[84,88] Furthermore, the high specificity observed in histology is also compromised in cytology samples, especially exudative effusions, due to more frequent nonspecific membranous

staining.[84,88–90] Thus, interpretation of positive results in cytology should be restricted to cases with unequivocal strong intensity membranous staining in tumor cells, keeping in mind that this also reduces the sensitivity (~16%−30%)[84,88] and limits the utility of EGFR-mutant ICC as a screening tool.

ICC for PD-L1 Expression in NSCLC

PD-L1 IHC has entered routine clinical practice to select patients for targeted immunotherapy but remains fraught with numerous challenges pertaining to the validation of 5 different predictive biomarker IHC assays in five concurrent clinical trials that tested five different drugs using variable cut-offs for positivity.[3,91,92] Of these, the Dako 22C3 pharmDx assay and the Ventana SP263 IHC assay are companion diagnostics, used with cutoffs of ≥50% and ≥1% staining in tumor cells, to determine eligibility for first-line and second-line pembrolizumab therapy, respectively.[92]

While CBs are commonly used for ALK, ROS1 and other genetic testing across various centers, PD-L1 testing on CBs or other cytology preparations is not yet widely practised. PD-L1 expression is known for its spatial heterogeneity, thus raising concerns about sampling bias when using small biopsies or cytology samples for PD-L1 testing.[92] A number of studies have analyzed the concordance of PD-L1 ICC in comparison with matched histology samples (Supporting Table 5). Cytology–histology discordance was higher in tumors that showed a greater degree of PD-L1 expression heterogeneity on the corresponding resection blocks.[93] For similar reasons, paired lung aspirates appear to yield better concordance with resections[26] compared with paired effusions or washes, as do paired cyto-histologic samples obtained from the same anatomical sites.[26,94] The temporal heterogeneity of PD-L1 (ie, increased expression with advanced stage)[95] may explain a higher positivity of cytology samples at progressed stage compared with the original resection specimens.[94] Thus, paired samples obtained concurrently or within a short interval show better correlation of PD-L1 scores.[94,96] Despite these limitations, most PD-L1 ICC studies have found good correlation with histology scores, with excellent interobserver agreement[26,97,98] and good intraobserver reproducibility,[93,98] particularly at the clinically relevant cutoffs of <1%, ≥1%, and ≥50% of tumor cell staining. In a recent report, pembrolizumab was administered to 11 patients based on high PD-L1 expression (TPS ≥50%) in CBs. No alternate histology sample was available in these patients. Eight of these patients showed objective clinical response to immunotherapy, with some having stable disease for over a year, similar to the clinical response profiles seen in patients selected based on IHC. This study offers the first clinical evidence of the predictive value of PD-L1 ICC of cytological specimens in lung cancer patients.[71]

Preparations—While most studies have used only CBs, Noll et al[58] compared matched fine needle aspirate (FNA)-derived CBs and direct smears versus small biopsies, and this study with 38 cases found a greater concordance of PD-L1 scores between alcohol-fixed Papanicolaou-stained DSs and small biopsies (97%) compared with CBs (82%). Munari et al[98] compared 55 FNA derived direct smears with paired resections and found excellent correlation at ≥50% TPS cutoffs. Jain et al[46] compared LBC (SurePath, Becton Dickinson, Franklin Lakes, New Jersey) with small biopsies and observed 88% concordance using a

cutoff of ≥25% for positivity. This study also included 5 matched DSs that showed a lower concordance of 60% with small biopsies. Despite limited studies on the latter, CBs and DSs both appear feasible for PD-L1 testing,[91,98] and CBs, in some instances, may be preferred over small biopsies.[94,99] Examples of PD-L1 ICC of non-CB and CB specimens are shown in Figures 3 and 4.

Fixatives—Large studies that used nonformalin preservatives including CytoLyt,[71,95,96] NovaPrep (Novacyt, Vélizy-Villacoublay, France),[97] or ethanol[98] do not specifically report any adverse effect on PD-L1 staining. The best membranous staining for PD-L1 has been observed in PreserveCyt⁻fixed Cellient CBs compared with formalinor CytoLyt-fixed CBs.[100] Jain et al,[46] in their study using CytoRich Red–preserved SurePath LBC preparations, observed aberrent nuclear staining for PD-L1 in one of their false negative cases.

Antibody clones and platforms—Studies have evaluated the 22C3 PharmDx assay on the Dako autostainer platform,[3,58,71,95,101] and the SP263 on the Ventana BenchMark Ultra.[98] Skov et al[93] demonstrated excellent concordance (95%) between the 22C3 and 28–8 clones on the Dako autostainer on CBs. Ilie et al[97] successfully validated 2 laboratory-developed assays on CBs using a 22C3 antibody concentrate across the Dako Autostainer and Ventana BenchMark Ultra staining platforms against the standard 22C3 pharmDx Assay.

Minimum number of tumor cells for PD-L1 ICC—While a minimum of 100 viable tumor cells for PD-L1 IHC has been considered as requisite for PD-L1 ICC, some studies report that scoring concordance is independent of cellularity,[58] and ICC may be performed in CBs containing fewer than 100 tumor cells without any significant reduction in concordance with histology scores.[93,97] Nevertheless, considering the inherent difficulties in ICC interpretation as discussed in the following paragraph, testing should be avoided in samples with less than 100 tumor cells.

Challenges—In histological specimens, PD-L1 staining positivity is defined as complete circumferential or partial linear cytoplasmic membrane staining of tumor cells of any intensity. Only cytoplasmic staining in tumor cells is not considered positive for scoring purposes. In non-CB cytological specimens, however, membranous staining is less distinct, since the cell membranes are intact and are not cut, as in FFPE tissue sections (Fig. 3). Thus, PD-L1 staining of the horizontally oriented cell membrane mostly appears as a diffuse surface staining mimicking cytoplasmic staining.

Overestimation of PD-L1 positivity due to nonspecific cytoplasmic staining of background macrophages and inflammatory cells can occur in both CBs[93,101] and smears[58] and can be particularly challenging in effusion samples with predominantly singly lying tumor cells admixed with inflammatory cells and mesothelial cells.[93,97] A confirmatory immunostain such as TTF-1 and/or a pan-leucocyte marker (eg, CD45) performed on a corresponding section will aid in confirmation of tumor cells for scoring of PD-L1 positivity.[58]

CONCLUSIONS

Cytology specimens are undoubtedly underutilized for predictive ICC testing.[102] With most laboratories (especially in the United States) restricting ICC to only CB preparations, a significant proportion of non-CB preparations that are more versatile and easily available remain unused. One of the main factors discouraging ICC on these preparations is the uncertainty of the validity of results due to practical difficulties in standardization. Based on our experience and a review of the literature, it is clear that with proper optimization and rigorous quality control, high-quality staining can be achieved on non-CB preparations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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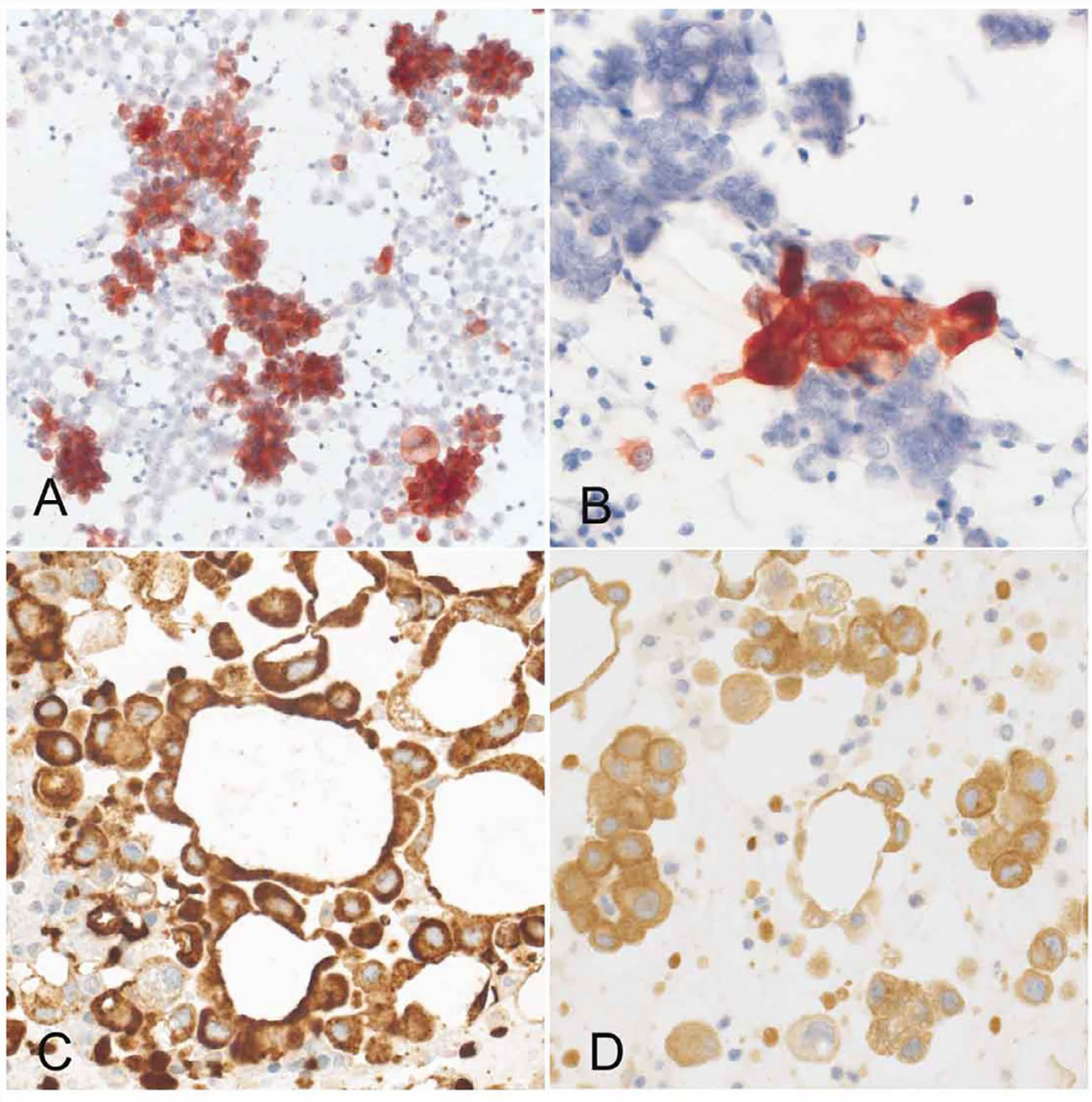

Figure 1.

ALK immunocytochemistry images of *ALK* re-rearranged pulmonary adenocarcinomas on previously Papanicolaou-stained non-CB cytology. (A, B) Homogeneously positive tumor cells with admixed ALK-negative benign cells. A laboratory-developed test using 5A4 antibody (Novocastra) was performed on an automated immunostainer (Leica Bond), and AEC (3-amino-9-ethylcarbazole) was used as a chromogen (A, magnification ×200; B, magnification ×400). (C, D) CB specimen stained with the Ventana ALK (D5F3) CDx Assay (C, magnification ×400) and ALK 5A4 antibody (D, magnification ×400) on the Ventana BenchMark system using 3,3'-diaminobenzidine (DAB) as a chromogen.

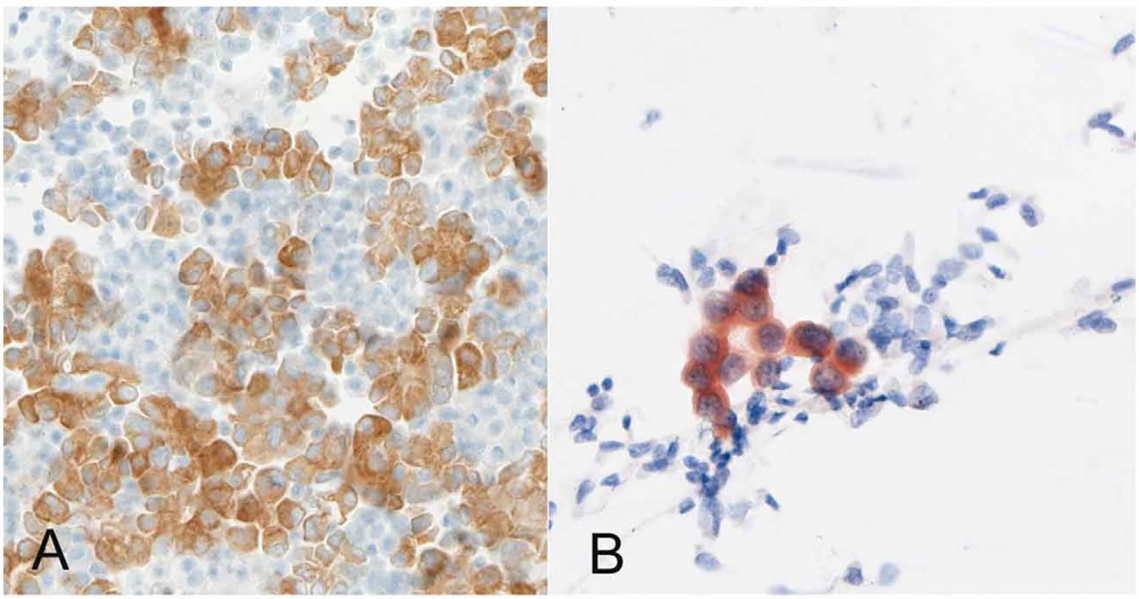

Figure 2.

Two immunocytochemistry images of *ROS1* re-rearranged pulmonary adenocarcinomas using D4D6 antibody (Cell Signaling). (A) Cell block specimen on the Ventana BenchMark system using 3,3'-diaminobenzidine (DAB) as a chromogen (magnification ×400). (B) Cell block specimen on previously Papnicolaou-stained non-CB cytology using Leica Bond system and AEC (3-amino-9-ethylcarbazole) as chromogen (magnification ×400).

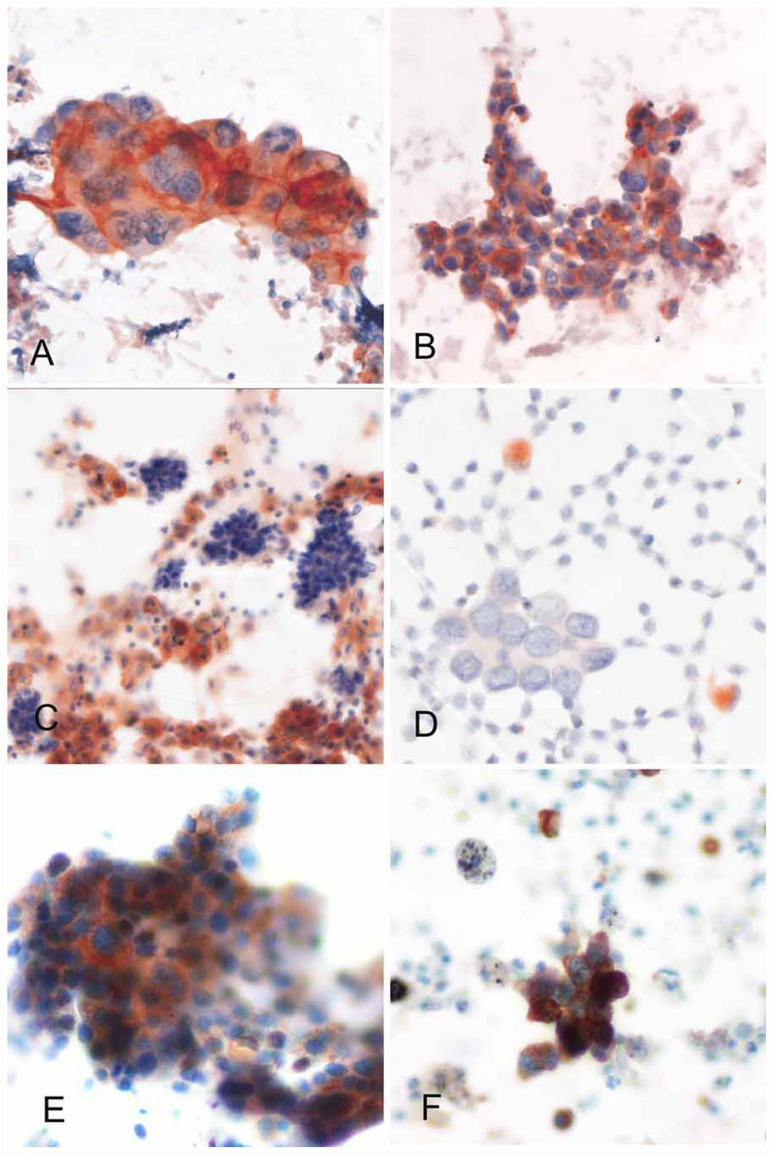

Figure 3.

PD-L1 immunocytochemistry images of non-CB cytology specimens. (A, B, E, F) NSCLC with almost all tumor cells positive for PD-L1. Both membranous as well as diffuse staining is evident. (C, D) PD-L1–negative tumor cells with macrophages serving as an internal positive staining control. (A-D) Laboratory-developed test using concentrated SP142 antibody on Leica Bond. (E, F) SP263 IHC assay on Ventana Benchmark XT (E, magnification ×400; F, magnification ×200).

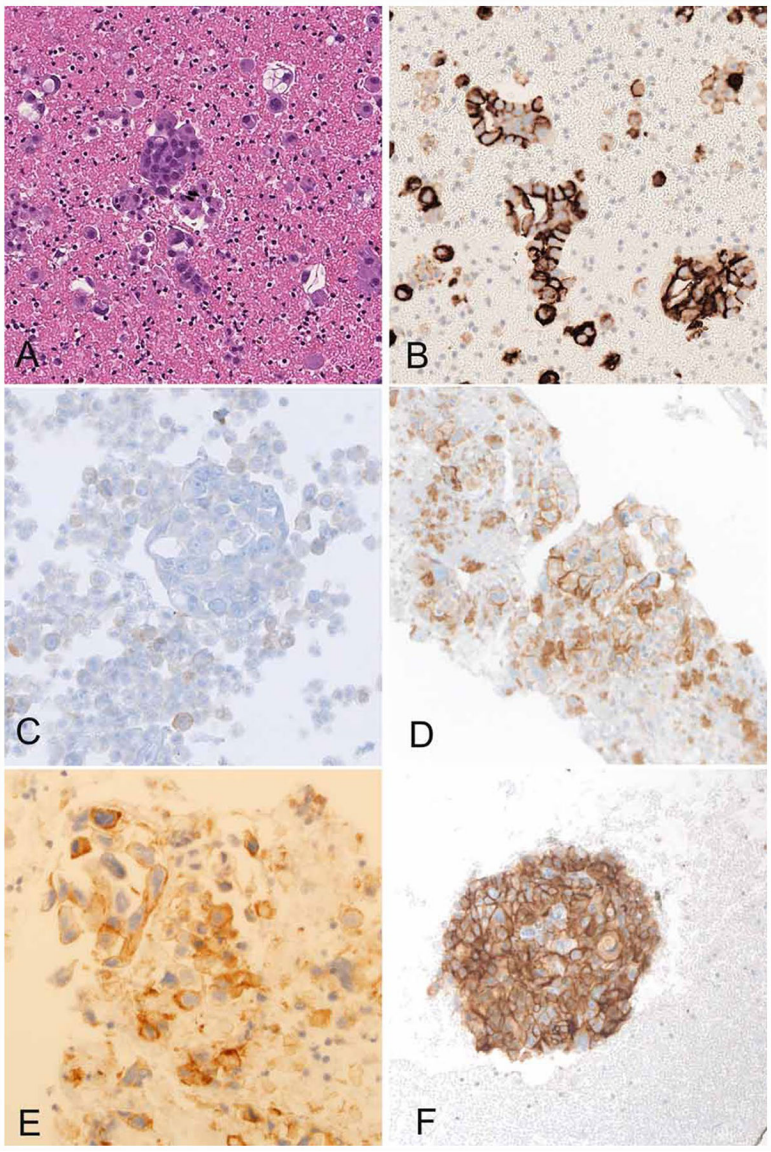

Figure 4.

PD-L1 immunocytochemistry images of CB specimens. (A, B) NSCLC with all tumor cells being positive for PD-L1 (hematoxylin and eosin and PD-L1, magnification ×200). (C) PD-L1–negative aggregate of adenocarcinoma cells and adjacent histiocytes, some of which are weakly PD-L1–positive (magnification ×400). (D) NSCLC with most tumor cells being PD-L1–positive (magnification ×200). (B-D) Laboratory-developed tests using DAKO 22C3 on Ventana BenchMark. (E, F) PD-L1–positive NSCLC by Ventana PharmDx Assay on BenchMark (magnification ×400).

TABLE 1.

Predictive Genetic Biomarkers in Lung Cancer

Molecular Alterations[a]	Recommended Method of Detection	Role of IHC as a Surrogate Predictive Biomarker
EGFR mutations ("hot spot" mutations with ≥1% prevalence)	Any molecular method with ability to detect mutations in histology or cytology samples with ≥20% tumor cells within a turnaround time of 10 working days	Not appropriate for treatment selection
ALK rearrangements	Cytogenetic (FISH) or IHC[b]	Appropriate for treatment selection
ROS1 rearrangements	Molecular (RT-PCR or sequencing) or cytogenetic (FISH/ISH)	Appropriate for initial screening
BRAF (p.V600E and non-p.V600E mutations)	Molecular (sequencing with evaluation of at least exons 11 and 15)	Not defined as yet
MET alterations (exon 14 skipping mutations, amplification)	Molecular (RNA-based assay confirmatory); FISH is widely used for amplification but no specific cut-off validated	Not defined as yet
RET rearrangements	Molecular (sequencing preferable to targeted RT-PCR) or cytogenetic (FISH)	Not defined as yet
ERBB2/HER2 mutations	Molecular (sequencing, particularly for exon 20 alterations)	No role for IHC[c]
KRAS mutations[d]	Molecular (targeted analysis of hot spots in codons 12, 13, 61, and 146)	No role for IHC

Modified from Lindeman et al.[2] Abbreviations: *ALK*, anaplastic lymphoma kinase; *BRAF*, V-raf murine sarcoma homolog b; *EGFR*, epidermal growth factor receptor; ERBB2/HER2, human epidermal growth factor receptor 2 gene; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; ISH, in situ hybridization; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; MET, mesenchymal epithelial transition receptor tyrosine kinase; NGS, next-generation sequencing; RET, rearranged during transfection; *ROS1*, ROS-associated oncogene 1; RT-PCR, reverse transcription–polymerase chain reaction.

[a] *EGFR*, *ALK* and *ROS1* alterations must be tested in all patients with advanced NSCLC for receiving approved targeted therapy; *BRAF*, *MET*, *RET*, *HER2*, and *KRAS* alterations should be tested only in extended gene panels for inclusion of patients who are wild-type for *EGFR*, *ALK* and *ROS1* in clinical trials.

[b] IHC has been found to be predictive of tumor response to crizotinib even in FISH-negative cases.

[c] Protein expression by IHC is not predictive of therapy response.

[d] *KRAS* testing may be performed as a single gene assay and if positive, may serve to exclude the patients from extended panel gene testing. Targeted therapeutic regimens for *KRAS* mutations have not shown clinical benefit.

TABLE 2.

Advantages and Disadvantages of Various Cytology Preparation Methods for Application of Immunocytochemistry

Preparation (Sample Feasibility)	Advantages	Disadvantages
CB (all types of samples except those with low cellularity)	Morphology and epitope stability comparable to histology	Inconsistent cellularity
	Multiple sections for ICC panels	Time-consuming
	Easier validation using IHC protocols	Different methods of preparation lacking standardization
	Histology FFPE controls acceptable (if collected in formalin)	Non-formalin-fixed CBs require revalidation
Direct smears (FNA, brushings, sedimented fresh effusions)	Bulk of the material used in cytopathology practice	Require larger quantities of antibody to cover entire slide[16]
	Excellent cytomorphology	Limited ICC panels
	Extra smears easily made during aspiration	ICC revalidation required
	ICC can be performed on the same diagnostic smear	
	Applicable even for smears with few tumor cells[a]	
	Coverslipped alcohol-fixed Papanicolaou-stained smears show stable epitopes for ~2 years[36]	
Cytospin specimens (low-volume fluid samples and FNA collected in nonfixative solutions, ideally 0.9% NaCl solution)	Rapid	Not suitable for specimens with blood or mucous
	Several slides from the same specimen for ICC panels[16]	Inferior cytomorphology to smears[b]
	Alcohol-fixed, air-dried, unstained cytospins can be stored at 4°C for a month, at −20°C for several months, and at −70°C for years without loss of antigenicity[16]	Cell counting to make aliquots of 0.5–1 × 10^5 cells/mL for optimum cellularity[16]
		ICC revalidation required
LBC (all types of cytology specimens)	Uniform cell collection and standardized processing	Altered cytomorphology[c]
	Clean background due to clearing of blood and mucous	ICC revalidation required
	Multiple slides from same specimen for ICC panels	
	Smaller quantity of reagent required	
	Material easily stored for up to 6–8 weeks in preservative solution at room temperature[37]	
	Alcohol-fixed, air-dried, unstained LBC slides can also be stored at −70°C for years without loss of antigenicity[16]	

Abbreviations: CB, cell block; FNA, fine needle aspirate; ICC, immunocytochemistry; IHC, immunohistochemistry; LBC, liquid-based cytology.

[a]Use of tumor cell mapping either by photocopy of the slide or marking location on a superimposed glass slide, or by saving microscope co-ordinates helps in relocating rare tumor cells on the ICC slide.

[b] Alteration of cell composition by speed and time of centrifugation can occur. We recommend careful centrifugation of 2 min at 400 rpm, followed by fixation in ethanol-acetone for 10 minutes.

[c] Cells more dyscohesive with shrinkage artefacts leading to difficulty in identification of isolated small atypical cells on low power examination.