To Obtain More With Less

Cytologic Samples With Ancillary Molecular Techniques—The Useful Role of Liquid-Based Cytology

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• ***Context.***—**Fine-needle aspiration cytology has been increasingly used as the first tool in the evaluation of several diseases. Although cytology has a relevant role in the discrimination between benign and malignant lesions, conventional slides cannot lead to 100% conclusive results. It was hoped that the introduction of liquid-based cytology (LBC) would improve the efficacy of cytology through standardization, quality improvement, and the possibility of carrying out ancillary techniques on the residual stored material. In recent decades, the application of genomic alterations has been studied on cytologic samples with feasible and reliable results. The molecular analysis offers a powerful aid to define the best clinical or surgical approaches and follow-up for patients. In recent years, the application of different ancillary techniques has been carried out on conventional slides even though LBC represents a useful additional and alternative method for molecular testing.**

Objective.—**To demonstrate the relevance of LBC as a valid aid to overcoming the difficulties encountered in the application of ancillary techniques on conventional slides.**

Data Sources.—**We examined and reviewed our experience with the application of ancillary techniques on LBC performed on different body sites.**

Conclusions.—**We emphasize that LBC achieves significant and accurate results. It represents a valid method for cytologic evaluation and it provides highly reproducible and informative molecular yields.**

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The application of ancillary techniques has become an integral part of the management of several tumors.[1–3] In fact, the knowledge of the molecular mechanisms that are linked to the development of tumorigenesis and cancer can be translated into clinical practice as an additional tool for diagnosis, therapy, and prognosis.[4–6]

In the last few decades, much evidence has suggested that the results of molecular analysis on fine-needle aspiration specimens improve the accuracy of cytologic diagnoses and guide patient management.[1–7] For example, many entities, such as lung cancers, are diagnosed mostly on the cytologic specimens, which represent the unique source of material to provide appropriate management and/or treatment. The possibility of obtaining adequate diagnostic material from small biopsies or cytologic specimens has also been facilitated by the introduction and development of minimally invasive procedures. Even though the morphologic evaluation is the cornerstone for the diagnostic interpretation of samples, it does not completely solve all diagnostic issues, especially in the field of cancer. For this reason, molecular analyses and evaluations of mutations and other genomic alterations have been integrated in the cytologic workflow of several neoplastic lesions.[1–9] Furthermore, the application of ancillary techniques on cytologic material has contributed to the reduction of both costs and issues to obtain good DNA/RNA quantity. Cytology provides the best alternative source because of well-preserved DNA, which is readily extractable and reasonably stable (from 6 months to 5 years) using all the different cytologic preparations (ie, freshly prepared, unstained direct smears, alcohol-fixed Papanicolaou [Pap], air-dried Diff-Quik smears).

In fact, the need for high-quality DNA/RNA is the main and critical point for the application of ancillary techniques. Several papers have demonstrated that ancillary techniques can be performed on formalin-fixed samples with good results because of the good preservation of cellular and architectural patterns of tissue. However, formalin is also associated with some important flaws, including interference with the degradation of nucleic acid and cross-linking action, which imply structural damage and fragmentation in the process of DNA extraction.[5–9]

Furthermore, different authors and papers[1–9] have investigated and questioned the variability in the DNA yield and quality obtained from differences in cytologic preparation, fixation, mounting media, and staining. Dejmek et al[6]

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Advantages and Limits of Liquid-Based Preparations	
Advantages	**Limits**
All the material in the vial	Not cost-effective in the short term
Standardized procedure (skip the smearing step)	Higher technical work
Hands-off technique	Resulting artifacts
Decreased screening area	Impossibility of rapid on-site evaluation
Decreased obscuring material	
Lack of air-drying effects	
Monolayer surface	
Well-preserved cells	
Stored material for further investigations (ie, ancillary techniques)	

demonstrated that the different fixation methods used for cytologic material provide better DNA quality when compared with histologic preparations. Additionally, they highlighted the comparative analysis of CytoLyt (Hologic Co, Marlborough, Massachusetts), PreservCyt solution (Hologic), and CytoRich Red (Hologic) collecting fluids. These authors demonstrated that CytoLyt solution resulted in a 5-fold higher DNA yield than CytoRich Red.[6]

Another critical point is represented by the use of different mounting media. Whereas Pertex is a xylene-based medium, Eco Mount is polymer based, showing better DNA yield. The type of fixation, represented by either ethanol-fixed or spray-fixed samples, does not alter the accuracy of molecular results. Otherwise in their analysis of staining, Dejmek et al[6] underlined that May Grunewald Giemsa had poor results whereas Pap-stained or even archived Pap-stained slides obtained adequate and well-preserved DNA extraction. Nonetheless, Killian et al,[10] collating Diff-Quik– and Pap-stained samples, demonstrated the significantly higher DNA molecular weight of Diff-Quik– than of Pap-stained cells from the same needle aspirate. Malapelle et al[8] compared the extraction between liquid-based cytology (LBC) stored material and laser capture microdissection on the LBC Pap-stained cells. The authors emphasized the value of laser microdissection, especially in cases with low cellularity. In another paper,[9] the same authors established that the use of more sensitive nonsequencing methods (high-resolution melting analysis Quik fragment and Taq-Man assays) could reduce the need for microdissection.

In our experience, we have found that both direct sequencing and real-time polymerase chain reaction can be appropriately performed on LBC stored material.[4,5] According to the literature, different authors[4,5,8,9] have established that 50 to 100 cells is an adequate amount for obtaining good polymerase chain reaction results. These latter studies clearly highlighted the equal or superior performance of LBC.

In fact, at the end of the 1990s, LBC gained popularity as an alternative method for collection and preparation of cytologic samples, including gynecologic and aspiration samples.[1–11] The LBC method is characterized by the preservation of cytologic material onto a liquid-based medium and then processing of the material with a semiautomated or fully automated system. A perusal of literature shows that it has been largely adopted all over the world, so that in several laboratories, especially in industrialized countries, it has replaced conventional slides (CS).[10–19] However, the introduction of LBC raised some conflicting opinions, especially concerning its diagnostic efficacy. There are several advantages in the adoption of LBC in terms of cost-effectiveness, standardized aspiration technique, hands-off approach, time savings, and simple application of ancillary techniques (both immunocytochemistry [ICC] and molecular analysis) for up to 3 to 4 months after the initial collection of material[8,10–19] (Table). On the other hand, LBC does not allow for a rapid on-site evaluation, the exact evaluation of tumor content in the solution, or the assessment of background material such as tumor diathesis. Additionally, the sample is characterized by a reduction of background, shrinkage in cell size, and fragmentation of clusters and is not conducive for tests that need unfixed material, such as flow cytometry (FC) and cellular culture[9–17] (Table). The fact that FC, which has a central role in the distinction between reactive hyperplasia and non-Hodgkin lymphoma (including low- and high-grade lymphoma), cannot be performed on LBC material might be partially overcome by the application of ICC on LBC.[20]

Among the different LBC systems, ThinPrep (TP; Hologic Co., Marlborough, Massachusetts) and SurePath (Becton, Dickinson, and Company, Franklin Lakes, New Jersey) are the most common methods used for both gynecologic and nongynecologic material.

After its introduction in our institution, we started to apply LBC (specifically TP) to exfoliative and nongynecologic aspiration cytology, with significant advantages in different body lesions, including thyroid nodules. Not only did LBC show a valuable role in the morphologic interpretation, but it also proved that the application of ancillary techniques is feasible and highly reproducible, with informative molecular results.[10,11,13–23] Ancillary techniques are applied on the remaining LBC material stored in the PreservCyt solution at room temperature. These techniques can be performed when the remaining material is at about 2 mL eluted in a minimal amount (ie, 5 mL) of PreservCyt solution.

Nonetheless, a perusal of the literature demonstrates that 2 of the major issues with respect to LBC are the development and validation of standard and univocal criteria (preanalytical, analytical, and postanalytical) for the interpretation of molecular testing on cytologic samples.[5,6] However, as reported by Rossi and Schmitt,[5] a definitive standardization is not fully completed. Nevertheless, in this review article we emphasize the role of LBC in the investigation of specific genetic alterations in cytologic samples of different entities. The evaluation of genomic alterations offers the advantage of obtaining an accurate knowledge of the molecular mechanisms of different oncogenic drivers and developing tailored targeted therapies in different malignant lesions.

THYROID

Thyroid cytology represents an important field for the application of the LBC method.[6,18–30] In fact, different

authors[10–15,31] have demonstrated that the morphologic artifacts of LBC do not limit the recognition of cellular and architectural features in the diagnostic categories regardless of the different thyroid classification systems. Moreover, several studies[18–28] show the usefulness of LBC in the application of several ancillary techniques for diagnostic and prognostic purposes and tailored management. These studies,[18–23,25–30] including some papers from our group, emphasize that the application of ancillary techniques (eg, ICC and molecular analysis) on LBC might be more easily performed because of the quality of the sample and the cellular preservation.

In the field of ICC, several authors* have confirmed that Hector Battifora mesothelial 1 (HBME-1) and galectin-3 show the highest specificity and sensitivity in diagnosing malignant lesions on LBC samples, although neither of these markers is so highly accurate to be recognized as the specific marker of malignancy. However, other authors[33–42] have emphasized the limitations and flaws of ICC (eg, low specificity), and have focused on the role of molecular testing as an additional aid to properly classify thyroid indeterminate nodules.

In recent decades, it has been emphasized that molecular alterations of specific pathways play a pivotal role as possible markers of malignancy in some specific variants of thyroid cancers. Importantly, some of these alterations arise early in the tumorigenic process.[33–39] For instance, papillary thyroid carcinoma (PTC), the most common thyroid malignancy, may carry v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), Ret proto-oncogene in PTC *(RET/PTC*), or rat sarcoma viral oncogene homolog (*RAS*) mutations.[33,34] A large number of prospective and retrospective studies have demonstrated the diagnostic role of somatic mutations and showed the high specificity of some of them (ie, *BRAF* mutations) as markers of cancer.[33,39]

We extensively studied and demonstrated the role of TP to ensure adequate and valid material for molecular analysis.[19–23,39] The specific and technical details about its applications on LBC stored material are described in our previous papers.[11,13,18,19,21] DNA extraction is performed on TP stored material using the QIAamp tissue kit (Qiagen, Hilden, Germany). The percentage of disease cells for molecular analysis is at least 50% in all TP samples. We assess the quantity and quality of the DNA spectrophotometrically (E260, E260:E280 ratio, spectrum 220–320 nm; Biochrom, Cambridge, United Kingdom) by separation on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, California).[11,13,18,19,21]

However, in a few selected cases, we also obtain adequate DNA from the microdissection of neoplastic cells directly from the LBC slides. As shown by Reynolds et al,[43] mutation analysis using next-generation sequencing can be successfully performed on residual cell pellets derived from LBC samples. We have also started to perform the same application of NGS on our thyroid LBC samples (E.D.R. et al, unpublished data).

The majority of our studies have dealt with the evaluation of the diagnostic and prognostic role of the $BRAF^{V600E}$ mutation carried out on TP samples obtained from thyroid fine-needle aspiration cytology (FNAC) specimens (Figure 1). Concerning the prognostic role of $BRAF^{V600E}$ mutation, it is clearly underlined that this somatic mutation correlates with extrathyroidal extension, advanced tumor stage at presentation, and lymph node or distant metastases.[21,33,35] Based on this evidence, $BRAF^{V600E}$ mutation is likely to play a critical role mainly for a possible more extensive surgical treatment (ie, total thyroidectomy or central lymphadenectomy).[21,33,35]

In fact, in a series of 50 LBC thyroid lesions diagnosed as positive for malignancy (PM), we found that $BRAF^{V600E}$ mutation was associated with multifocality of cancer ($P < .001$) and nodal involvement ($P < .001$).[21]

In another paper, somatic mutations represented a valid diagnostic aid for the diagnosis of suspicious for malignancy. Because of the high specificity of $BRAF^{V600E}$ mutation, our mutated suspicious for malignancy specimens were diagnosed as PM.[22] The feasible molecular application of *BRAF* analysis on TP was also encouraged by Chang et al,[27] who reported 84.9% sensitivity in the PM specimens.

Furthermore, we also successfully demonstrated that $BRAF^{V600E}$ mutation can be detected with the monoclonal antibody against the mutated *BRAF* protein (VE1) on either LBC samples or histologic specimens. In our analysis of VE1 on TP stored material, we showed that VE1 had a high diagnostic accuracy among malignant lesions.[19] Specifically, we found that 51 of 55 malignant cases showed concordant VE1 expression on both cytology and histology, and VE1 expression was also significantly correlated with $BRAF^{V600E}$ mutation. In fact, we demonstrated a statistically significant relationship between molecular expression and VE1 positivity ($P < .001$) in PTCs.

In this series, the highest sensitivity and specificity (ranging between 80% and 100%) were obtained with a cutoff of 2+ as a positive VE1 test.[19] In fact, all cases with 2+ and 3+ VE1 expression (Figure 2) were $BRAF^{V600E}$ mutated. However, even though some drawbacks in the evaluation of VE1 expression are ascribed to the adoption of a semiquantitative (number of VE1-positive cells and intensity of VE1 expression, as previously discussed[19]) evaluation, we concluded that VE1 represents a feasible first-line approach for $BRAF^{V600E}$ analysis.

According to the literature, although $BRAF^{V600E}$ represents 95% of all *BRAF* mutations, uncommon *BRAF* mutations (Figure 3) are also found in thyroid lesions, and they are mostly associated with the follicular variant of PTC (FVPC).[39] Therefore, we analyzed uncommon *BRAF* mutations by DNA methods on TP thyroid samples and demonstrated diagnostic and prognostic correlations between uncommon *BRAF* mutations and 106 TP thyroid lesions with a malignant histologic diagnosis of FVPC.[39] First, we confirmed the accuracy of LBC as a valid diagnostic tool for the evaluation of uncommon *BRAF* mutations, and second, we found 100% correlation between FVPC and uncommon *BRAF* mutations.[39] We did not find any uncommon *BRAF* mutations in the PTCs; additionally, 3 of 6 cases with uncommon *BRAFs* had a cytologic diagnosis of follicular neoplasms. We found that uncommon *BRAF* mutations were associated with the histologic diagnosis of encapsulated FVPC, whereas $BRAF^{V600E}$ mutation was found among infiltrative FVPCs. This yield confirmed the lower malignant potential of encapsulated FVPCs, as recently established in their reclassification as noninvasive follicular thyroid neoplasm with papillary-like nuclear features. According to the seminal paper by Nikiforov et al,[44] this new entity is defined by a specific set of morphologic features characterized by a follicular growth pattern with nuclear features of PTC, scant nuclear pseudoinclusions, and

* *References 11, 12, 15, 18, 25, 28, 31, 32.*

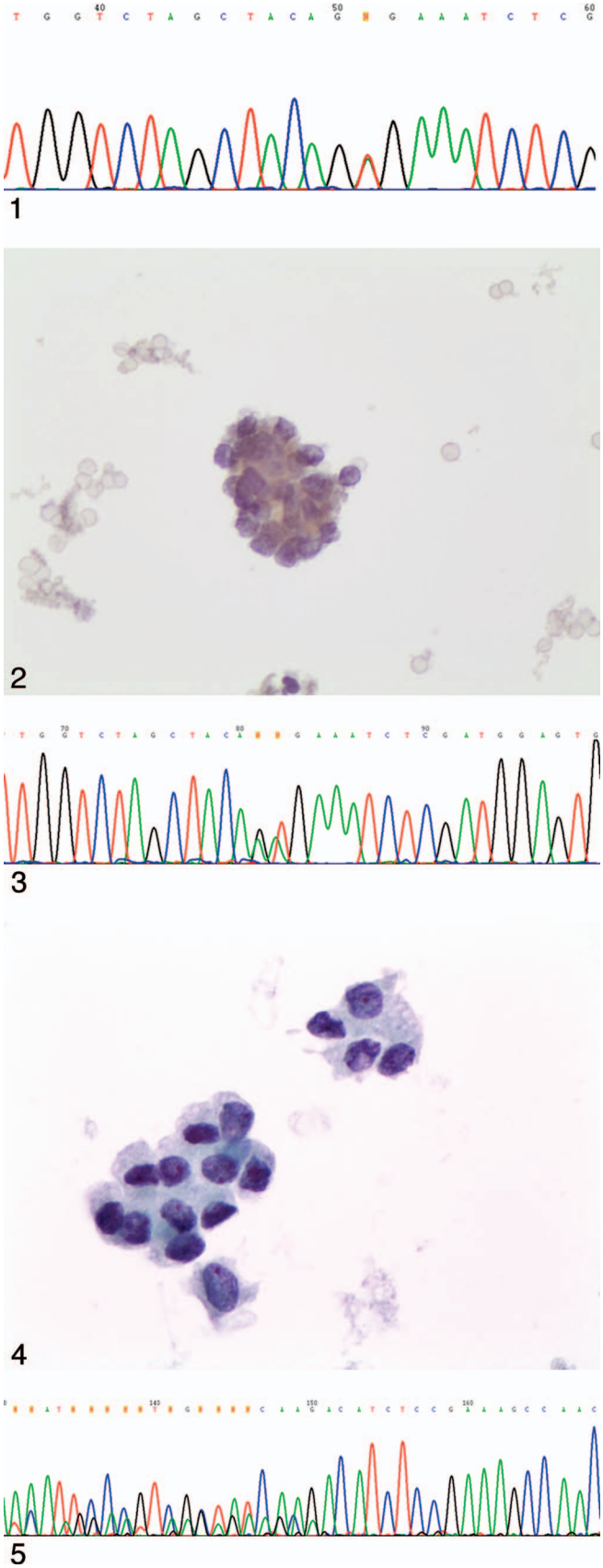

Figure 1. *Sequence of* p.V600E *in a thyroid lesion diagnosed as positive for malignancy on liquid-based cytology.*

lack of papillary structures and psammoma bodies. Additionally, the molecular analysis performed by Nikiforov et al[44] demonstrated that their noninvasive follicular thyroid neoplasms with papillary-like nuclear features did not harbor *BRAF*^{V600E} mutation and mostly had *RAS* or other mutations.

In our laboratory, we perform *RAS* mutations on TP stored material obtained from wild-type *BRAF*^{V600E} cases. However, we do not report a high statistical significance of *RAS* mutations for malignancy so that we do not routinely perform them in our molecular protocol for thyroid lesions.

Additional research from our group demonstrates the recognition of peculiar morphologic features associated with *BRAF*^{V600E} mutation in LBC thyroid samples[40,45,46] (Figure 4). As described in our paper, the evidence of specific morphologic findings of *BRAF*^{V600E} mutation represents a new foreseeable diagnostic and predictive sign of mutation also on FNAC, regardless of cytologic categories or classification systems.[40,45,46] As previously documented by Finkelstein et al[41] and Virk et al[42] on histologic thyroid samples, we found that the presence of *BRAF*^{V600E} mutation was associated with some specific morphologic features on TP thyroid samples diagnosed as PM. These features included the identification of cells characterized by eosinophilic large "plump" cytoplasms and nuclear features of PTC (plump cells), but also the presence of malignant cells characterized by smaller and eccentric located nuclei (so-called sickle-shaped nuclei), which were recognized in all the cases that harbored *BRAF*^{V600E} (Figure 4) but were not present in either wild-type *BRAF* or uncommon *BRAF* cases.[45,46]

Recently, the role of microRNAs (miRNAs) has been evaluated on cytologic and histologic thyroid lesions.[23,47–51] As discussed in our recent paper, we documented that the application of miRNA analysis on LBC stored material can be an additional and useful tool to achieve the correct diagnosis.[23] In recent years, the majority of authors have referred to miRNA evaluation mainly on histologic samples of thyroid tumors.[47–51] Nonetheless, a few papers have focused on the excellent results of miRNA expression in thyroid FNAC processed with different cytologic methods, including LBC.[23,47–51] To the best of our knowledge, we were the first group who studied a prospective series of 60 TP cases, including 27 follicular neoplasms, with the application of a miRNA panel. For the evaluation of miRNAs, the total RNA is isolated with miRNeasy Mini Kit (Qiagen, Milan, Italy) from TP stored material following the manufacturer's instructions.[23] We studied several miRNAs, including miR-375, miR-10b, miR-92a, miR-221, miR-222, and U6 snRNA.

Among the 5 analyzed miRNAs, MiR-375 was overexpressed in all malignant follicular neoplasms and in 95% of

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Figure 2. *Strong expression of VE1 on liquid-based cytology in a case positive for malignancy with* BRAF *mutation (hematoxylin-eosin, original magnification ×200).*

Figure 3. *Sequence of* p.V600K *mutation in a case of thyroid lesion diagnosed as suspicious for malignancy on liquid-based cytology.*

Figure 4. *Details of the morphologic features of plump cells and sickle-shaped nuclei on liquid-based cytology obtained from a "positive for malignancy—favoring papillary thyroid carcinoma" case (hematoxylin-eosin, original magnification ×200).*

Figure 5. *Evidence of* EGFR *mutation (deletion 19) in a sample of non–small cell lung cancer on liquid-based cytology.*

the PM specimens. These yields demonstrated that it might be used as a valid adjunct to rule out benign lesions and to support malignancies such as PTCs and/or FVPCs.[23]

In conclusion and according to our experience, we underline the valuable and feasible role of LBC in empowering the diagnostic and prognostic value of thyroid cytology. This evaluation may guide and change the clinical and/or surgical management of patients with thyroid neoplasms.

HEAD AND NECK

Fine-needle aspiration cytology is used as the first and most important tool for the evaluation of lumps in the head and neck area, including salivary gland lesions.[52–59] In the last few decades, in our institution, FNAC has been adopted as the first approach for the evaluation of salivary gland lesions.[52] In the field of salivary lesions, the high diagnostic accuracy of FNAC is partially limited by some diagnostic controversies, mainly due to the overlapping morphologic features among the different entities.[52–55] These morphologic controversies are notable and critical in either CS or LBC methods, especially in those samples obtained from tumors with biphasic components.[52–55]

Despite the well-known advantages of LBC, its adoption for salivary gland cytology is controversial, mostly because of artifacts in cellular architecture, extracellular material, shrinkage of cells, reduction of the inflammatory components, and stromal background. Specifically, extracellular material and inflammatory components play a central role in the diagnosis of several neoplasms. Nonetheless, as noted by Rarick et al[53] and Parfitt et al,[54] the coupled analysis of both LBC and CS offers some additional and valid insights, especially if cytopathologists are familiar with LBC artifacts.

In our recent study, which included 1729 salivary gland FNAC specimens (analyzed with both LBC and CS), we estimated an overall specificity of 97.6% and a diagnostic accuracy of 91.3%, which were not influenced by cytologic methods.[52] Nonetheless, some authors[56–59] point to the valid application of ancillary techniques on cytologic samples to reduce some morphologic drawbacks and pitfalls. A recent review article by Griffith et al[58] discussed the clinical significance of the detection of chromosomal rearrangements with fluorescent in situ hybridization (FISH) and ICC. However, the same authors analyzed the application of ancillary techniques only on CS and/or cell blocks from salivary gland lesions. Additionally, Pusztaszeri and Faquin[56] and a few other authors[57,58] dealt with the analysis of these genetic alterations exclusively on CS, and none of these papers mentioned their use on LBC.

In our institution, we have a limited daily experience with ICC or molecular analysis on LBC salivary material, and we carry out ancillary analysis in selected cases of neoplasms characterized by basaloid features.

Furthermore, some authors[56,58] have noted that malignant salivary tumors are driven by specific somatic mutations, including gene fusion, which can be studied by parallel RNA sequencing as well as whole-exome gene sequencing. A review article by Pusztaszeri and Faquin[56] discussed the currently known tumors with their harbored genetic alterations and the specific translocations discovered in a subset of salivary gland tumors. Allegedly, a few specific genetic alterations are also associated with benign lesions. In fact, a specific translocation t(3;8)(p21;q12) involving *PLAG1* and *CTNNB1* (the gene encoding β-catenin) can be found in 50% to 60% of pleomorphic adenomas. However, some specific translocations are present in malignant salivary neoplasms: the t(11;19)(q14-21;p12-13) translocation in 60% to 70% of mucoepidermoid carcinomas, the t(6;9)(q21-24;p13-23) translocation in 64% (28%–86%) of adenoid cystic carcinomas, and the t(12;22)(q13;q12) translocation in 85% of hyalinizing clear cell carcinomas.[56,58] Hence, Griffith et al[58] demonstrated that the t(12;15)(p13;q25) *ETV6-NTRK3* translocation, specifically associated with mammary analogue secretory carcinoma, can be reliably detected on a cell block processed from FNAC.

However, despite the feasibility of performing molecular testing on LBC samples, it seems that the majority of the studies underline that ancillary techniques are better performed on the cell block material.[53–55,58] This is because some biomarkers were validated with the use of tissue blocks.

Human papillomavirus (HPV)–related head and neck squamous cell carcinoma represents one of the most frequent malignancies in the lingual and palatine tonsils.[58,59] According to the literature, about 50% of these patients present with a head and neck mass with initially unknown primary site. The identification of HPV can be achieved with different HPV testing, including p16 ICC, identification of high-risk HPV DNA, by in situ hybridization, and other amplification methods.[59] Several studies[58,59] have found that p16 ICC is frequently performed on LBC in order to diagnostically discriminate HPV-related carcinomas from those that are not viral carcinomas. Specifically, the recognition of HPV can lead to defined tailored therapeutic approaches. Few studies underline the role of liquid-based assays for head and neck squamous cell carcinoma; however, these assays are a valid diagnostic method, especially in those cases with minimal material.[59]

LUNG LBC CYTOLOGY

Frequently, the discovery of lung cancer triggers a complex diagnostic workup in which cytology plays a central role as the first diagnostic tool. Not only does FNAC lead to the correct diagnosis, but it also provides specific clues for the most appropriate and tailored therapies.[60,61]

In the past decades, the role of cytology was emphasized by the increased use of minimally invasive diagnostic surgical approaches, such as endobronchial ultrasound-guided biopsy, which are able to provide suitable material for (1) the diagnosis and staging of lung cancers, especially in patients with metastatic or locally advanced tumors, and (2) the application of ancillary techniques.[60–62] Nonetheless, the guidelines from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology recommend the evaluation of molecular testing on cell blocks, even though that may change with the new guidelines, which suggest evaluation also on direct smears.[63] In fact, despite these recommendations, at many institutions the evaluation has been carried out on both CS and LBC.[8,9,60,61,64–68]

Specifically, several papers have demonstrated both (1) the adequacy of material for molecular testing and (2) the advantages of the cytologic application of ICC and gene mutational testing (ie, epidermal growth factor receptor [*EGFR*] or *KRAS* analysis).[8,9,67–69]

Nevertheless, the acknowledgement of the molecular pathogenesis of lung cancers has maximized the role of targetable genomic alterations, which could lead to tailored

therapies especially, but not only, for metastatic lung cancers.[60–70] Actually, current recommendations propose that the analysis of molecular targets be carried out through polymerase chain reaction–based methods and/or FISH on lung cytology.[65,66]

The functional activations of *EGFR* (Figure 5) and anaplastic lymphoma kinase (*ALK*) are predictive of the therapeutic response to targeted agents such as tyrosine kinase inhibitors.[60,61] In addition to these driver alterations, tumors are also tested for Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation, which is associated with resistance to tyrosine kinase inhibitors. Recently, a fourth target *C-ROS* oncogene 1 (*ROS1*) gene rearrangement has seemed to provide drastic clinical responses to targeted crizotinib.[63] Despite the fact that only *EGFR*, *ALK*, and *ROS1* currently represent the standard target of care, lack of targetable genomic alterations of these genes can lead the analysis of less common driver mutations. For example, among them, an emerging role is linked with *BRAF, NRAS,* v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*), neurotrophic tyrosine kinase receptor 1 (*NTRK1*), negative regulator of glucose-repressed gene 1 (*NRG1*), and fibroblastic growth factor receptor (from *FGFR1* to *FGFR4*) amplifications.[60,61,63]

Additionally, new immunotherapy agents in lung cancer lead to some critical evaluation. In fact, these agents involve checkpoint inhibitors that need the assessment of programmed death ligand-1 (PD-L1) expression by ICC for patient selection. These checkpoint inhibitors interrupt the PD-L1 binding with its receptor, programmed death receptor-1 (PD-1), on the surface of cytotoxic T cells. Many tumor cells can upregulate the expression of PD-L1 and evade the natural immune response. The evaluation of PD-L1 can be performed on cytologic samples (including LBC) using monoclonal anti–PD-L1 antibody, and then it can support the use of pembrolizumab in the treatment of patients with advanced non–small cell lung cancer.[71]

All of these analyses can be performed on cytology regardless of the different preparations. In fact, numerous authors[8,9,64–68] emphasize that the molecular profile of lung tumor can be studied on cytology material, including CS and LBC. Concerning LBC, molecular testing in lung cytology is likely to maximize the efforts to obtain adequate cellular material and to preserve the stored material for either molecular testing or additional cell blocks for ICC.[8,9,20,67,68] However, a few papers have focused on the use of LBC lung samples for the application of molecular testing. For example, Petrella et al[72] demonstrated that LBC preparation led to sensitive and highly reproducible molecular yields, showing a diagnostic accuracy as high as that reported in CS. Wu et al[73] analyzed a series of 434 LBC lung samples and found similar results, even though these authors reported a lower rate of concordance with the histologic samples. Recently, Bellevicine et al[67] retrieved 362 lung cases, including 204 LBC and 158 CS. In their assessment, CS showed a higher DNA yield and was more frequently cell rich, even though the differences in mutation rate between CS and LBC did not have a statistical significance.

Da Cunha Santos et al[68] demonstrated that *EGFR* mutations can be detected by FISH and chromogenic in situ hybridization methods**.** However, some authors found that the use of the Colorado criteria for the interpretation of FISH results is suboptimal for the evaluation of *EGFR* gene copy number on FNAC**.** Moreover, Savic and Bubendorf[69] provided an overview of the role of multitarget FISH in the diagnosis of lung cancer and clarified some equivocal cytologic findings. In their evaluation, these authors[69] concluded that FISH is the gold standard for the identification of *ALK* rearrangements and treatment with the *ALK* inhibitor crizotinib. They also compared the accuracy of *ALK* FISH with ALK ICC on cytologic specimens of non–small cell lung cancer. It is important to underline that the performance of ALK ICC largely depends on antibody clones (5A4 versus D5F3), signal detection system, and scoring system. Specifically, this comparative analysis resulted in only 1 false-negative and 1 false-positive non–small cell lung cancer on ALK ICC. The sensitivity, specificity, and positive and negative predictive values for ALK ICC were 93.3%, 96.0%, 93.3%, and 96%, respectively.[70]

In our experience, we have confirmed that the use of different cytologic methods, including LBC, does not affect DNA or RNA quality.[61] Specifically, Smouse et al[61] and Rossi et al[62] emphasize that all the different cytologic preparations are a reliable source for the detection of *EGFR*, including the less frequent *EGFRs*, and *KRAS* mutations in lung cancer.

In another series, we also performed *EGFR* mutational analysis (ie, mutations from exons 18–21) on LBC samples obtained from 28 metastatic mediastinal lymph nodes in non–small cell lung cancer. The evaluation was accurately performed and yielded 100% conclusive results that led to specific tailored therapies for the patients. The results clearly described the central role and efficacy of LBC in the management of metastatic lung cancers.[20]

In conclusion, our experience and the data from the literature demonstrate that LBC is an effective method for the application of molecular testing. In this perspective, the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology guidelines should include and suggest the performance of ancillary techniques on LBC storage material as a comparable alternative option.[63]

LYMPH NODES

The cytologic evaluation of enlarged and suspected lymph nodes has been recognized as a suitable diagnostic tool for both nonneoplastic and neoplastic diseases.[74–79]

In recent decades, numerous papers have highlighted the central role of cytology in the diagnoses of reactive lymphoid hyperplasia, infectious and granulomatous lymphadenitis, and primary or metastatic malignancies.[74–79] Some authors mention high diagnostic accuracy in the cytologic diagnosis of lymph nodes processed with LBC.[20,76–79] Whereas some authors[72] underline the feasible application of ICC and molecular testing (ie, receptor gene rearrangements or chromosome translocations) on LBC for diagnostic and predictive purposes, other researchers[80] have drawn different conclusions, mostly because of the inability of phenotyping by FC.[72,79] However, in their studies, Ahmad et al[74] and Nasuti et al[75] had 4.5% and 15.7% of lymphomas diagnosed on FNAC with the application of FC and/or ICC, respectively.

In our personal experience, we evaluated a series of 263 lymph node samples processed with the LBC method and analyzed with the application of ancillary techniques. Whereas in our 4 (3.7%) suspicious for lymphoma cases the cytologic diagnosis was achieved only on the morphologic samples because of scant stored material for ICC and

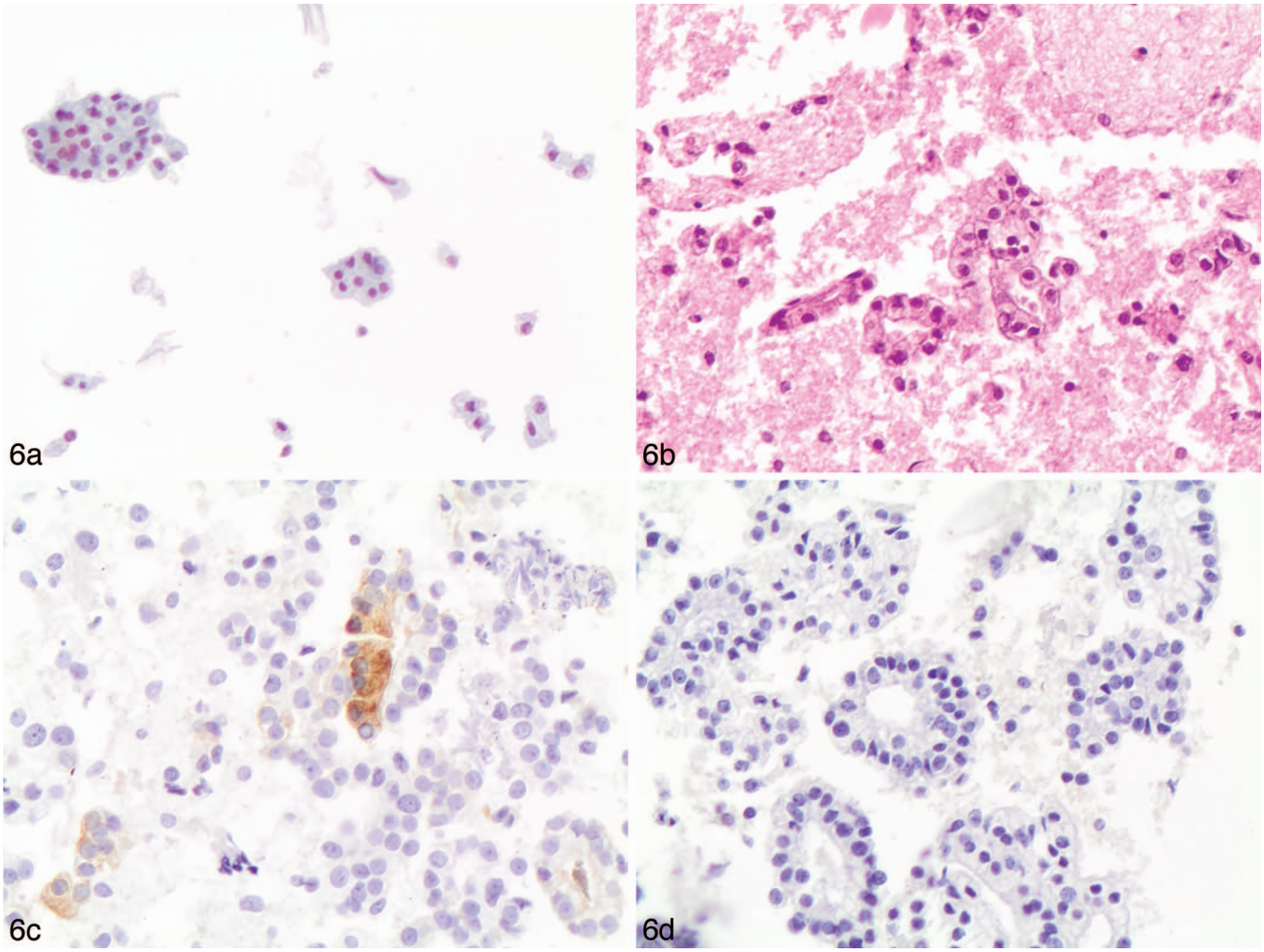

Figure 6. *a, Cytologic features of a lymph node metastasis from a prostate adenocarcinoma on liquid-based cytology (LBC). b, Cell block sample obtained from LBC for the same case. c, Positivity of neoplastic cells for prostate-specific antigen on cell block. d, Negative thyroid transcription factor 1 in metastatic cells on cell block (Papanicolaou stain, original magnification ×200 [a]; hematoxylin-eosin, original magnification ×200 [b]; avidin-biotin-peroxidase complex, original magnification ×200 [c and d]).*

the inability to phenotype by FC on LBC, in some primary unknown neoplasms either ICC (Figure 6, a through d) or molecular analysis (ie, receptor gene rearrangements or chromosome translocations) led to conclusive results.[20] In a paper published by our group, the evaluation of the immunoglobulin heavy chain gene rearrangement on LBC stored material supported the diagnoses of lymphoma in pancreatic and peripancreatic abdominal masses.[80]

In conclusion, we encourage the use of LBC in the cytologic evaluation of lymph nodes, especially in those cases in which morphology alone is unable to achieve a conclusive diagnosis. In those cases, the application of ancillary techniques on LBC samples is likely to contribute to a correct outcome and triage of patients. Some authors underline that the inability of phenotyping by FC in suspicious for lymphoma cases is likely to be overcome by the advantages of ICC application together with gene rearrangement studies on LBC samples from lymph nodes.

CONCLUSIONS

In recent decades, LBC has gained popularity as a reliable diagnostic method in the field of cancer. The ease in the application of ancillary techniques and NGS technology maximize the possibility to analyze tumor genetics with a minimal amount of high-quality DNA/RNA. The advantages of LBC are ascribed mostly to its reliability and minimal skill requirements, which may suggest that it can be preferred over CS. Unequivocally, our application of ancillary techniques in different neoplastic entities clearly assessed the relevant and central role that LBC methods are assuming in the cytologic scenario.

Our overview of the application of molecular methods for diagnostic and prognostic purposes in the diagnosis of different cancers emphasizes that LBC achieves significant and accurate results and represents a valid method in the cytologic evaluation because of its high reproducibility and 100% informative ICC and/or molecular yields.

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