EXPERT REVIEWS

The diagnostic and prognostic role of liquid-based cytology: are we ready to monitor therapy and resistance?

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Here, we evaluate the diagnostic and prognostic role of liquid-based cytology (LBC) in different body lesions, including thyroid, lung, effusions and malignant breast lesions. LBC has gained consensus after being applied to both non-gynecologic and fine-needle aspiration cytology. Although some remain sceptical regarding the diagnostic efficacy of LBC, mainly when used alone, in recent years, good results have been obtained as long as it showed a high diagnostic accuracy. Here, we discuss the additional possibility of storing material for the application of ancillary techniques (immunocytochemistry–molecular analysis) with several diagnostic and prognostic advantages, which may pave the way for the challenging evaluation of both monitoring responses to treatment and resistance to targeted therapies in thyroid, lung, breast carcinoma or malignant effusions. Furthermore, it provides the use of several molecular spots as specific targets for personalized therapy.

KEYWORDS: effusions • immunocytochemistry • liquid-based cytology • malignancy • molecular analysis • nodular lesions • targeted therapies

Fine-needle aspiration cytology (FNAC) is the first and worldwide diagnostic procedure applied in the field of both palpable and deep body lesions [1,2]. It has found a widespread application because of its simplicity, safety and cost–effectiveness leading to a correct diagnosis concerning the nature of the lesions in >70% and to a correct clinical approach in > 90% of patients regardless of the site [1]. To some extent, conventional cytology (CC) has been the cornerstone method for many years even though it copes with some important drawbacks represented by: the unpredictable rate of inadequate samples and the diagnostic difficulties in defining the overall efficacy and specificity in some diagnostic samples. Based on the figures of inadequacy and false-negative results, CC was openly discussed and criticized in some specific contexts including mainly cervical cytology, breast, lung and thyroid lesions [3–6].

The introduction in 1996 of liquid-based cytology (LBC) has gained enthusiasm as a new technique for collection and preparation of cytological specimens, first in cervical cytology and then with the application on FNAC with reliable and feasible results [1,7,8]. The two most common US FDA-approved methods for processing the cytological samples use an alcohol-based fixative solution. In the first method (ThinPrep5000™, Hologic Co., Marlborough, MA, USA), the cells are aspirated from a methanol-based solution (Cytolyt™), then filtered and transferred onto a positively charged slide with a gentle positive pressure. This method was characterized by a collection of cells into a methanol-based preservative solution then processed with a fully or semi-automated device reducing the background interference from blood, inflammatory, debris and cells overlapping.

In the second method, the cells are collected in an ethanol-based solution (CytoRich™), centrifuged twice then slowly sedimentated onto a poly-L-lysinated slide and eventually

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stained with a specific hematoxylin–eosin stain (SurePath™, TriPath Imaging, Burlington, NC, USA). The final result for both methods is one slide for each lesion where all cells are concentrated in a thin layer on the central area of the slide measuring 20 mm^2 for ThinPrep and 13 mm^2 for SurePath. Apart from their specific characteristics, we did not find any significant difference in the cytological evaluation, which may invalidate a correct interpretation of the lesion. Furthermore, LBC introduction improved the diagnostic efficacy in several cytological steps through procedure, standardization of method, sample quality improvement, screening support, speedup and quality control [7,8]. The growing interest in the new cytological preparation raised the well-known conflicting opinions and controversial data regarding the efficacy of LBC even if several aspects in terms of cost–effectiveness, time-sparing and first and foremost in the easy application of ancillary techniques such as immunocytochemistry (ICC) and molecular biology must be underlined [1,7–11]. We aim to exploit some of its important advantages that are related to the assessment method, including the reduction of inadequate samples, better fixation of cells, the clean and purified background that enhances the nuclear details of lesions even though a training is advisable for a complete knowledge of all the main differences with conventional cytological preparations.

Nevertheless, one of the additional major advantages of LBC is the feasible use of the residual material for the extraction of high-quality DNA/RNA even after a long period of storage [7–11]. Inasmuch as cancer therapy is becoming more and more targeted, LBC may represent a promising and feasible method for approaching the knowledge of genetic changes in cancer and their tailored treatment in a presurgical phase [9].

Thyroid cytology

Thyroid morphology

The cytological evaluation of thyroid lesions represents the first and widely accepted screening method for the diagnosis of thyroid nodules with high sensibility and diagnostic accuracy, which is counterparted by lower specificity [1,2]. Taking into account the distribution of thyroid lesions, approximately 65–70% are benign, 5–10% are malignant while the remaining 25–30% represent a 'gray zone' in which different follicular entities are diagnostically included [1,2,12–15]. The attempt of deciding on the extent of surgery in a case of indeterminate cytology remains a vexing question and a difficult challenge. In fact, this latter category includes several subcategories (follicular neoplasm [FN], suspicious for malignancy and atypical cells of undetermined significance) that are not always accurately defined, bearing a different risk of malignancy and different surgical treatments with a quite high rate of unnecessary thyroidectomises and an expected 20–30% rate of malignancies [11–15]. The evaluation of morphology alone could create some misunderstanding and pitfalls so that several authors encouraged the use of ancillary techniques as sensitive markers in providing estimated risk of malignancy [1,2,8–11,16–19]. To some extent, the application of LBC provided the possibility to easily carry out these techniques with material stored in the preservative solution.

Ancillary techniques on thyroid cytology

Literature data show that several papers have been set out on the use of ICC in thyroid FNAC without any regard to the cytological method used [1,2,8–11,20]. In fact, some data support a feasible ICC application on cell blocks yielding both 100% sensitivity and specificity, as well as Cochand-Priolett *et al.* reckoned 100% sensitivity, 85.2% specificity and 100% negative predictive value with anti-mesothelioma antibody 1 (HBME-1) and cytokeratin 19 [11,20,21]. Eventually, utilization of an ICC panel may be the best choice as highlighted by some authors and by Rossi *et al.* stating that HBME-1 and Galectin-3 were able to discriminate between low and high risk of malignancy in FNs with 92% overall diagnostic accuracy in concordant positive ICC cases [22]. Rarely does the detection of positive ICC prove to be more than a suggestion of malignancy while the high specificity of molecular detections, classified as 'rule-out' or 'rule-in' tests, is a strong indicator of cancer, enabling physicians to accurately balance risk and reward in selecting a treatment and follow-up [2,17,19,23–25]. The challenging advances in molecular genetics of thyroid cancer have been applied for identifying new diagnostic markers of malignancy also on FNAC [2,17,19,23,24]. The diagnostic role of FNAC in recognizing malignancies is mostly represented by the identification of papillary thyroid carcinoma (PTC) and the assumption that *BRAF*, *RET/PTC* or *RAS* mutations are found in >70% of PTC also associated with a more aggressive tumor behavior [26–28]. In many reports, *BRAF* mutations, mainly involved in the activation of *MAPK* pathway, have been correlated with extrathyroidal extension, advanced tumor stage at presentation and lymph node or distant metastases with the final impairment of the function of the sodium iodide symporter and of other genes metabolizing iodide. All these processes are involved in the unfavorable prognosis of these patients [27–31]. Specifically, the association of $BRAF^{V600E}$ mutation with a poorer outcome on FNAC assumes a significant diagnostic role and provides valuable prognostic information for a preoperative risk stratification of thyroid lesions [27–30]. In this perspective, the role of *BRAF* mutations on FNAC has been assessed by several studies in which both DNA and RNA analyses were applied on CC or LBC as recently reported by Chang *et al.* [8–10,12]. Hence, Rossi *et al.* referred that the detection of $BRAF^{V600E}$ mutation in the suspicious for malignancy has a 100% histological correlation with a diagnosis of thyroid carcinoma ($p = 0.0353$), lymph-node metastases ($p < 0.0001$), extracapsular invasion ($p = 0.03$) and multifocality ($p = 0.0003$), envisaging a more aggressive surgical treatment [8]. From a diagnostic point of view, a comparison between ICC results and gene mutations in the FN category assessed that an ICC panel is more likely to achieve a diagnosis than *BRAF* analysis, especially due to the great percentage of follicular variant of PTC (FVPC) with high wild-type *BRAF* prevalence [19]. We reported 87.5% wild-type *BRAF* in the FNs diagnosed as FVPC on histology whilst

Nikiforov *et al.* and Ohori *et al.* reported a higher incidence of $BRAF^{V600E}$ mutation ranging from 25 to 33% FNs [8,19,26].

Hence, many authors agree that $BRAF^{V600E}$ mutation has been highly associated with PTC (Figure 1) and more often with the aggressive variants (e.g., tall cell and columnar variants) as well as some rarer and uncommon *BRAF* mutations are more frequent in FVPCs and linked with less aggressive behavior and/or encapsulated variants [31,32]. Although these rarer mutations induce partial kinase activity and alter amino acids which are essential for catalysis leading to an oncogenic *BRAF* activation [27], the need for prospective trials may extend the role of predictive significance of *BRAF* mutations to patients with low risk PTC or its variants as well as it has assessed the role in advanced metastatic radioactive iodine-refractory disease where patients may benefit from $BRAF^{V600E}$-targeted kinases. Since the evaluation of MAPK or other DNA pathways did not provide complete and exhaustive achievements, we reasoned that thyroid lesions, especially FN category, might be studied with miRNAs. These miRNAs are small endogenous, non-coding RNAs that are negative regulators of gene expression with a role in regulating cells proliferation, differentiation and survival [33–35]. Thyroid tumors have been associated with deregulation of different miRNA profiles including miR-221, 222 and 146b which seem to be mostly upregulated in classical PTCs, while the new promising miR-375 resulted in PTC, FVPC and also in medullary thyroid carcinoma [33–35]. Specifically, miR-375 upregulated expression may be a negative regulator of the expression of YAP1 (a growth inhibitor) and SLC16a2 (a transporter of thyroid hormone) and it may induce an aggressive behavior in terms of residual disease, lymph node and distant metastases and mortality. Especially in medullary carcinoma, which may benefit from the new frontiers of specific targeted therapies, miR-375 may accompany or even supplant conventional clinic–pathologic risk factors and this may open, as well as in breast carcinoma, to a prognostic and predictive significance in personalized tailored therapies based on the molecular yields easily carried out on LBC.

The evidence of patients harboring *BRAF*-mutated carcinomas, either resistant to radioiodine *de novo* or becoming refractory to radioiodine, supports the growing role of LBC in the management and monitoring of mutations for both therapeutic responses and resistance to therapy. The latter, which is explained by the association between $BRAF^{V600E}$ mutation and the loss of radioiodine avidity, may be sought and detected in the cytological phase and consequentially treated with MEK inhibitors. Few clinical studies carried out *in vitro* with genetically engineered mice demonstrated that inhibitors of MAPK signaling pathway may restore iodine uptake in $BRAF^{V600E}$ iodine-resistant carcinomas [35].

Lung cytology

Lung morphology

Lung cancer represents the first worldwide leading cause of cancer death and one of the major public health concerns [36]. Despite the increasing number of new cases per year and the need for screening programs, this malignant entity is more frequently discovered for symptoms or chest radiographs performed for other intents in many cases recognized as a locally advanced or metastatic disease with only a few of them eligible for surgical resection [36]. In the complex diagnostic workup, cytology (including exfoliative respiratory cytology) is often underutilized and underestimated as the first tool to obtain diagnostic material. This mainly happens due to the location of the nodule and the number of possible pitfalls and false positive/negative diagnoses without a conclusive cytological report [36]. Despite this evidence, the rediscovered role of cytology in patients with metastatic or locally advanced tumors has been enhanced by the increased use and excellent results of minimally invasive diagnostic procedures used for diagnosis and staging of lung cancer, such as endobronchial ultrasound-guided biopsy [37,38]. Its essential role was proven not only in achieving the correct diagnosis but mostly in leading to the application of molecular testing regardless of the cytological method [36].

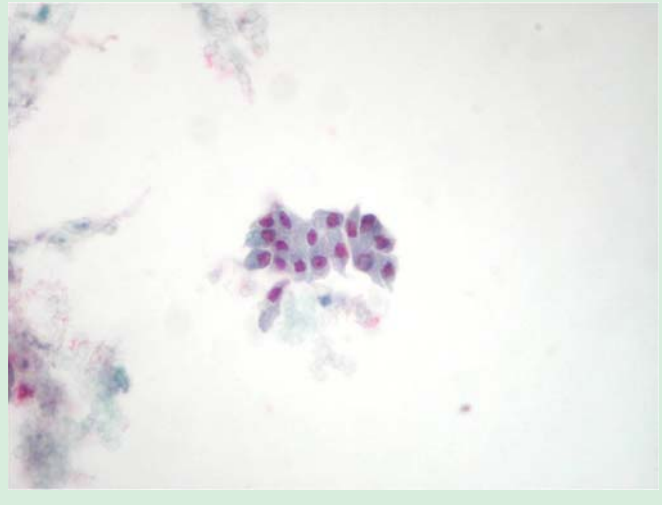

Figure 1. Details of the morphological features of cells obtained from a 'positive for malignancy-PTC' case on liquid-based cytology (40×).

Lung molecular insights

In the last two decades, increasing knowledge of the molecular pathogenesis of lung cancers has maximized the role of targeted therapies, especially, but not exclusively, for unresectable malignancies (Figure 2) [38–41]. Taking together these data, the discovery of the functional activation of EGFR as well as anaplastic lymphoma kinase (*ALK*) gene rearrangement seems to support both prognostic and predictive benefit for therapeutic response with some targeted agents such as tyrosine kinase inhibitors (TKIs) [38,39]. This represents a new challenge since, until few years ago, the central role of cytology had been mainly restricted to the morphological differentiation between small cell and non-small cell lung carcinomas without any possible further diagnostic or prognostic implication [40–45]. According to the latest recommendations of the international

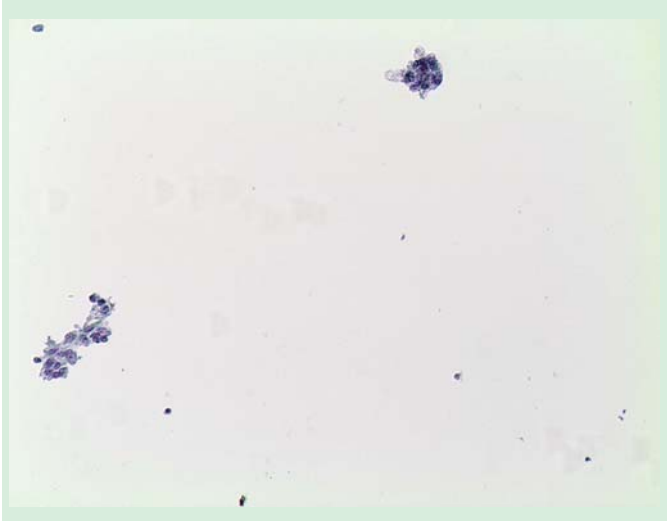

Figure 2. Details of the morphological features of lung adenocarcinoma (liquid-based cytology, 40×).

Association for the Study of Lung Cancer, the American Thoracic Society and the European Respiratory Society, all lung adenocarcinomas should be analyzed for *EGFR* and/or *KRAS* mutations [37]. Not only did review articles, original studies and guidelines appraise the feasible role of CC, but also they reported the equal efficacy of LBC when compared with CC and histological samples in providing outstanding material for gene mutational assays, essentially in the *EGFR* and *KRAS* genes [40–42]. Moreover, recently Malapelle *et al.* demonstrated that conventional smears showed higher DNA yields and were more frequently cell-rich than the LBC slides even though the difference in adequacy and in mutant *EGFR* rate between the two samples types did not reach a statistical significance [43,44]. Hence, the application of LBC does not affect DNA or RNA quality and molecular testing as confirmed by some studies involving both conventional and LBC techniques on FNAC and exfoliative samples [43–45]. The latter aspect seems to involve some limitations in the presence of an exfoliative respiratory cytological sample mainly due to the high proportion of benign epithelial cells, which may lead to inconclusive results. The precious quality of cytological preparations needs to be validated by large cytological series, which are often quite scant apart from the one from Billah *et al.* including a series of 209 cytological samples supporting and validating the use of cytological samples as a reliable source of *EGFR* and *KRAS* molecular detection in lung cancer [40].

It is known worldwide that *EGFR* mutations are detected in 10–40% of lung tumors and highly correlated with a selected group of patients, including women, never smokers and adenocarcinoma histology. Furthermore, 70–80% of patients demonstrated a better clinical outcome and disease-free survival rate when treated with first-line EGFR inhibitors, including gefitinib or erlotinib [36–39]. The majority of *EGFR* mutations (about 90%) are either short in-frame deletions in exon 19 or point mutations in exon 21 (p.L858R) caused by the alteration of ATP/inhibitor binding site, stabilizing the binding of the drugs and potentiating their inhibitory effects. On the other hand, other less common mutations (p.G719A in exon 18 and p.L861Q in exon 21) also increase susceptibility to TKIs [37–39,45,46]. On balance, we have to deal with the fact that not all activating mutations confer sensitivity to TKIs. In fact, some *in vitro* studies have demonstrated that mostly exon 20 *EGFR* mutations render the receptor less sensitive to the TKIs supported by the evidence that insertions/deletions and the substitution of p.T790M in exon 20 induce resistance to TKIs [37–39,45,46]. From this standpoint, the screening for all *EGFR* mutations can be applied on cytology not only to select specific treatments but also to detect primary or acquired resistance to TKIs. Our previous paper on cytological lung cancer samples analyzed for molecular testing (including common and rare *EGFR* mutations) led to our assessment that a mutation is detectable in small cytological samples with also low yield of genomic DNA so that the size and yield of the specimen are not necessarily limiting factors for mutational analysis [45]. Although the gold standard input level of DNA amount is validated in our everyday practice with 20% of neoplastic cells, the rate and amount of tumor cells higher than 20%, without any further subquantitative evaluation, did not influence our mutation detection [45]. Our second successful challenge in *EGFR* mutations detection on cytological as well as on histological samples was the detailed identification of rarer mutations, both primary and acquired, in exons 18 and 20 that can maximize the adoption of alternative and more 'personalized' tailored therapies in a very early phase of patient diagnosis. Eventually, the sensitivity of molecular application on FNAC material may vary a lot using different methods that seek the need to properly validate the molecular procedure in every laboratory. Cytological samples, including LBC, represent an effective alternative method with high DNA and RNA quality also in the close future perspective of obtaining outstanding cellular results for genome sequencing such as the application of next generation sequencing (NGS) also in the ample field of lung cancer [46]. Since then, the introduction of NGS has been only carried out with CC (both FNAC and exfoliative cytology) and based on scraping or laser micro-dissection as long as mutational profiles may be affected by the amount of specific DNA and the proportion of malignant cells versus possible contaminations with non-tumor cells. New series with NGS carried out on LBC samples need to be confirmed. Moreover, a recent paper by Buttita *et al.* demonstrated *EGFR* mutations, corresponding to those detected in histological samples, in 42% of the samples without cytopathological evidence of neoplastic cells highlighting the superior sensitivity of the NGS diagnostic approach and the possibility to use somatic mutations of frequently mutated genes in lung tumors (i.e., *p53, KRAS* and *EGFR*) for early diagnosis in fluid samples (sputum, broncho-alveolar lavage and pleural effusion) [47].

Effusions cytology

Serous effusions

Malignant cells found in serous effusions are significantly related to poor survival rates and, consequently, this finding is generally accepted as a hallmark of dismal prognosis [48].

Despite the increasing and growing interest in use of all the modern laboratorial resources in serous effusions examination, the principal approach is still characterized by the cytological evaluation of these effusions (including pleural, pericardial and peritoneal), which has not been completely assessed in all its potentialities in these past years [49–51]. Some recent studies reported that one-fifth of body's serous membrane effusions per year are malignant with approximately 50% as metastatic adenocarcinomas (Figure 3) followed by pulmonary large cell carcinomas and lymphomas/leukemia (about 15% each) [48–51].

The challenging implications of the presence of malignant cells in effusions highlight that an accurate cytological evaluation is the critical and mainstream diagnostic tool, mainly encouraged by its simplicity, safety and cost–effectiveness in reducing all the possible consequences and complications of a more aggressive biopsy procedure that often may even fall short of adequate diagnostic material [48–52]. In some cases, these serous effusions are the only available material for diagnosis, so that the recognition of maligant cells as well as the discrimination with reactive mesothelial cells need the identification of specific cellular findings regardless of the cytological methods which include conventional smears, cytospin, cell blocks and LBC [48,52–56].

Ancillary techniques on effusions

In this perspective, ancillary techniques, such as fluorescence *in situ* hybridization, chromogenic *in situ* hybridization, telomerase assays, comparative genomic hybridization and microarray-based comparative genomic hybridization, are carried out in order to ascertain an accurate diagnosis. Although different groups report good results with each of these techniques, we extensively use LBC as the leading method for the morphological evaluation of the cytological findings in effusions. Besides, an article by Hoda evaluated all the known literature for the morphological aspects of LBC in non-gynecological cytology as well as the accuracy of both Thin Prep and SurePath in the interpretations of effusions in alignment with the data from Ylagan [51,57]. Keeping these evidences in mind, we recently analyzed our 13-year experience with the application of LBC effusion preparation underscoring the pros and cons of this method. In detail, we reported 72 ovarian metastatic carcinomas in which WT-1 was not specific in ruling out the correct diagnosis, while CA125 combined with other markers (including keratin 7 and Calretinin) showed a good specificity in the discrimination with mesotheliomas. Moreover, the hotspot evidence that only 49 negative effusions were diagnosed as a malignant biopsy result (1.5%) underlined the high negative predictive value of LBC effusions [58]. A recent article published by Shah *et al.* evaluated the use of effusion fluid for NGS-based, multigene mutation profiling for personalized treatment [59]. Another recent paper by Davidson *et al.* identified vimentin and Zeb1 as markers of poor chemoresponse in metastatic serous ovarian carcinoma effusions and suggested NCAM as a potential prognostic marker of metastatic disease [60].

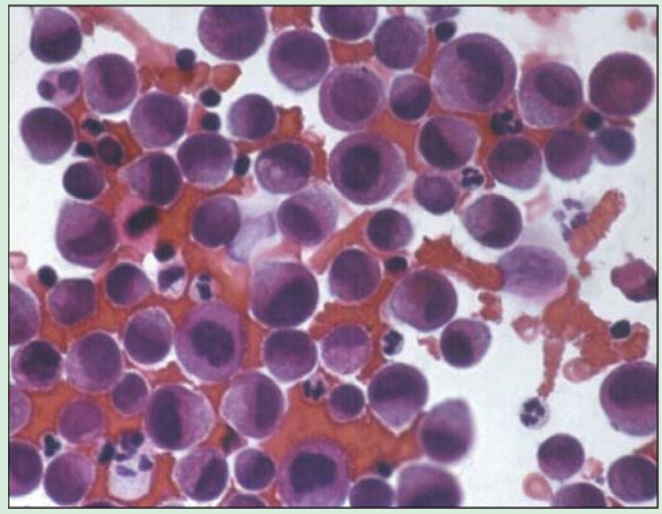

Figure 3. Details of the morphological features of peritoneal malignant effusion from a gastric carcinoma (liquid-based cytology, 40×).

Additionally LBC offered the strong diagnostic aid of the accurate application of ancillary techniques (both molecular testing and ICC) on effusion cytology ended up in a lucky application of these techniques for diagnostic, therapeutic and prognostic especially when LBC is adopted [48–51]. The microenvironment setting of malignant effusion is complex and composed of a plethora of different elements that comprise, among others, the cells and molecules from blood and lymphatic circulation, cells and structural components of serous membrane, and the malignant cells with their varied phenotypes and related molecular products [52]. Because serous effusions can be invaded by tumors originating in different anatomic regions, cytopathological investigation of these samples can offer different levels of morphological interpretation that encompass the identification of an unknown primary site of a malignancy, histogenetic discrimination of malignant cells (if certain malignant cells represent sarcomas, carcinomas or lymphomas), if the atypical cellular morphology represents malignant or benign alterations or if the malignant cells are metastatic or residents [53,54]. Despite lack of an unquestionable marker of malignancy until now, there are a number of monoclonal and polyclonal antibodies that can compose different immunocytochemical panels currently used to enhance the performance of cytological investigation. These panels are designed to recognize hormone products of malignant cells secretion, such as calcitonin, thyroglobulin, estrogen (ER), progesterone (PR) and others; or products that identify the structural components of the normal cells that originated certain malignancies, such as vimentin, keratins, melanin and others [55,56]. The possibilities are immense, and presently it is possible to address antibodies against a number of molecules that are involved with cellular metabolism, tyrosine kinase receptors, growth factors, interleukins and other players present in malignant effusions [60,61]. Not surprisingly, most of these markers are targetable for

therapeutic proposals, and ongoing efforts are now focused to improve not only diagnoses and prognoses, but cancer therapy as well [60].

Special attention has been dedicated to the advanced-stage ovarian carcinoma metastasized to serous effusions that is recognized as an aggressive malignancy with poor survival rates, frequently. The heat shock protein 90 (HSP90), which shows an anti-apoptotic activity, was recently tested by both Elstrand *et al.* and Shoshan's groups evaluating the role of HSP90 in 265 effusions from women with advanced-stage ovarian carcinoma [61–64]. They found that HSP90 nuclear expression was significantly higher in samples of women post-chemotherapy when compared to effusions from women pre-chemotherapy effusions ($p = 0.005$), and significantly related to previous treatment with both Platinol ($p = 0.024$) and paclitaxel ($p = 0.007$). Cytoplasmic HSP90 expression, on the contrary, was significantly higher in effusions from women with complete compared with incomplete/no response after second-line chemotherapy ($p = 0.016$). However, no association was found between HSP90 expression and other clinicopathologic parameters or survival. The authors concluded that HSP90 does not provide prognostic data in patients with advanced ovarian carcinoma effusions. Conversely, they proposed that HSP90 is a potential therapeutic target in this patient group, because HSP90 may benefit from treatment with HSP90 inhibitors to potentiate the effectiveness of Platinol and paclitaxel in patients with recurrent advanced ovarian carcinoma effusions through a possible interaction with glucose metabolism [61–64]. Antiangiogenic therapy is also a new paradigm for cancer patients with malignant serous effusions. The old premise that starving tumor is a potential and reliable option for cancer treatment has nowadays increased options of different strategies that merit careful attention [61]. Yano *et al.*, for example, elegantly evaluated this model of therapy. They studied malignant pleural mesothelioma, a tumor that is constantly refractory to the conventional chemotherapy and radiotherapy [65]. As angiogenesis plays a critical role in mesothelioma progression, the authors investigated the molecular pathogenesis and evaluated the efficacy of vascular targeting therapy in mesothelioma. They found that selective VEGF inhibitors were effective to treat mesotheliomas that highly express VEGF. In addition, they found that multiple kinase inhibitors (E7080), which potentially inhibit various angiogenic receptors, suppressed mesothelioma progression and prolonged survival in both high-VEGF-producing and low-VEGF-producing mesothelioma models. This is a remarkable finding because a number of different strategies with other molecules that modulate cancer progressions and survival present in malignant effusion microenvironment can be evaluated with very promising results [66]. There are many mechanisms of resistance to anti-VEGF therapy recognized at the moment; various malignancies are able to bypass the angiogenic obstruction [65]. However, there are promising improvements combining molecular players involved in metabolic response and hypoxia with anti-angiogenic armamentarium. The recent years witnessed increasing evidence associating the hypoxic and metabolic responses related to the tumor adaptation against anti-angiogenic therapy. The transcription factor hypoxia-inducible factors are recognized as the principal players that induce metabolic and phenotypic changes, including increased ability to facilitate invasion and metastasis [65]. The combination of anti-angiogenic agents with inhibitors of tumor hypoxic and metabolic adaptation is believed to be of great promise, as anticipated [67]. This combinatination has been innovatively reported for more than a decadebefore the cancer cell-enhanced glycolysis, measured by glucose transporter-1 investigation, has proved to be essential for the survival adaptation of tumor cells in effusions [67]. These findings have been later revised by Liao *et al.*, who postulated that increased expressions of glucose transporter-1 and carbonic anhydrase IX combination are indubitable parameter of malignant effusion [66]. Another way to escape anti-angiogenic blockage was studied by Fuang and colleagues who reported the effect of Endostar, a recombinant human endostatin expressed and purified in *Escherichia coli*, combined with an angiopoietin-2 specific inhibitor, L1-10, on mouse model with injected lung carcinoma cells in malignant pleural effusion. They demonstrated a significant synergistic effect on the formation of malignant pleural effusion and tumor growth; in addition, the authors also evinced that Endostar combined with L1-10 plays a role in the reduction of pleural inflammation and inhibition of tumoral angiogenesis and pleural hyper-permeability. The authors hypothesized that Endostar combined with L1-10 could complementarily act by reducing the local VEGF and local IL-6 and downregulating VEGF expression in pleural tumors [68]. The examples discussed so far are sufficiently robust to reveal the huge potential of serous effusion samples as a fascinating biological tool for therapeutic proposals.

Breast cytology

FNAC still has a role in the assessment of breast lesions, especially in the context of clinical and radiological benign nodules, symptomatic breast lumps and breast malignancies (local recurrences, metastasis, advance breast tumors in patients with poor clinical conditions and assessment of lymph node status) [69]. Nonetheless, the success of FNAC is highly dependent on the adequate collection and preparation of the cytological smears [70,71]. When there are no trained practitioners to adequately handle FNAs, the LBC technique may overcome problems related to ill-preserved specimens and poorly prepared smears [72]. Indeed, air-drying and spreading artefacts are significantly reduced in LBC preparations [72,73].

In general, LBC provides better cellular preservation, less cell overlapping and elimination of obscuring background elements when compared to conventional smears [72,74,71]. On the other hand, alterations in architecture and cellular morphology have been described in FNAC specimens processed with LBC and prior training in evaluation and interpretation of LBC specimens is necessary to avoid potential diagnostic pitfalls [72,73,75].

Architectural changes have been reported in LBC preparations, including fragmentation of cellular aggregates that results in cellular dissociation and smaller clusters [75]. One study showed that 3D arrangements were more pronounced in breast

lesions prepared as LBC specimens [73]. These architectural features – small clusters, loss of cohesion, 3D configuration – are potential sources for an erroneous diagnosis of malignancy. Usually, cell morphology is better preserved in LBC specimens with an enhancement of the nuclear detail, including the presence of well-defined nucleoli [73,75]. The myoepithelial cells (bipolar cells) are commonly decreased in number and some of them show an intact cytoplasm resembling fibroblasts or epithelial cells [72].

LBC specimens often demonstrate a clean background due to the elimination of obscuring elements such as blood, inflammatory cells and cellular debris [73–75]. However, an 'informative' background may also be eliminated, including the loss of mucous, stromal fragments and fat tissue.

As a result of the architectural and cell changes described above, some types of breast lesions prepared as LBC specimens present different cytological features in comparison to those found on conventional smears. In particular, the diagnosis of fibroadenoma seems to be the most problematic: small cell aggregates, enhanced cellular dyshesion coupled with prominent nucleoli, decreased numbers of myoepithelial cells and loss of stromal fragments are commonly found in LBC specimens and may potentially result in an erroneous diagnosis of malignancy [73–75]. Conventional smears and LBC preparations have comparable performance in detecting breast carcinomas [73,74]. Some studies observed a reduced size of neoplastic epithelial clusters in comparison to conventional smears [73–75]. Nuclear features such as hyperchromasia, less coarse chromatin and more prominent nucleoli were described by Ryu *et al.* in breast carcinomas processed by the LBC technique (Figure 4) [73].

Conventional smears and LBC present similar diagnostic performance for non-gynecological cytological specimens. One study that compared matched conventional smears and LBC preparations found 100% sensitivity for detection of malignancy in breast specimens for both methods [75]. However, in this study, the LBC technique showed a lower specificity rate (74%) for classifying benign breast lesions in comparison to conventional smears (93%) [75]. Another study that evaluated the LBC technique in 352 cases of benign and malignant breast aspirates found a few number of false-negative and false-positive cases, resulting in high sensitivity (97.7%) and specificity (98%) rates [75]. As previously discussed, prior training in interpreting LBC specimens is very important to achieve high diagnostic accuracy. In fact, one study showed a decrease in false-negative rate (from 12 to 4%) coupled with an improvement in sensitivity (from 86 to 94%) after LBC training [73].

Ancillary techniques on breast cytology

Specific cell receptors such as the hormonal receptors (ER and PR receptors) and the HER-2 are therapeutic targets in breast cancer. Therefore, the investigation of these receptors is mandatory in cases of invasive breast carcinoma. Hormone-positive tumors, for example, are usually responsive to hormonal therapies and have a better outcome [76]. When evaluated by the immunohistochemistry method, around 80% and 60–70% of invasive breast carcinomas are positive for ER and PR, respectively. The evaluation of hormonal receptors expression by immunohistochemistry can be performed in a variety of cytological preparations (conventional smears, LBC specimens and cell blocks) and the results are similar to those found in formalin-fixed and paraffin-embedded (FFPE) tissue specimens [77]. Leung and Bédard [78], for example, showed an overall accuracy of 97 and 89% for detection of ER and PR, respectively, in breast aspirates processed by the LBC technique. An important advantage of LBC versus conventional smears is the preservation of hormonal receptors antigenicity for long periods of time. One study showed that immunostaining for ER and PR was preserved on LBC slides stored at room temperature for up to 56 days [78].

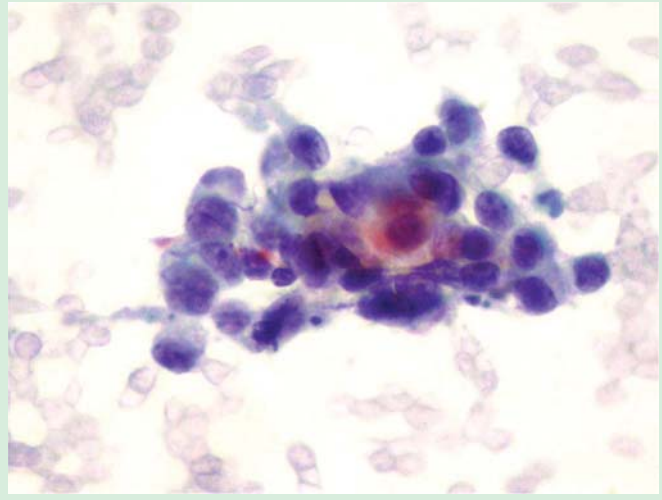

Figure 4. Details of the morphological features of breast ductal carcinoma (liquid-based cytology, 40×).

Approximately 15% of the patients with invasive breast cancer present amplification of the *HER-2* gene. Specific therapies against the HER-2 protein are recommended for those patients whose tumors are HER-2 positive, including protein expression scored 3+ by immunohistochemistry or gene amplification determined by *in situ* hybridization (ISH) techniques such as fluorescence ISH, chromogenic ISH or silver ISH [76]. Well-defined recommendations for the assessment of HER-2 status in breast cancer were established by the American Society of Clinical Oncology and the College of American Pathologists on FFPE breast material such as those obtained by core needle biopsies or excisional specimens [79]. Indeed, the results of HER2 immunohistochemistry assessment in cytological specimens are variable in the literature. Some studies showed a poor-to-moderate agreement of HER2 testing between breast aspirates processed by different cytological methods (conventional smears, LBC specimens) and FFPE tissue samples [80]. However, the assessment of HER2 status in cell block preparations from breast aspirates seems to be reliable and highly concordant rates were observed between these preparations and the FFPE standard tissue sections in recent studies [81,82]. In

contrast to immunohistochemistry, a good correlation between cytological and tissue section specimens has been demonstrated for the determination of HER2 status in breast cancer by ISH techniques. High concordance rates of HER2 status were found between LBC specimens and FFPE histological sections using fluorescence ISH and chromogenic ISH [83] techniques. A concordance rate of 96% between LBC specimens and histological material was achieved in a study that investigated the role of the dual silver ISH technique for the evaluation of HER2 status in a series of 47 invasive breast carcinomas [84].

We conclude that in spite of the peculiar cytological features that require appropriate training to avoid interpretative errors, LBC has similar diagnostic accuracy for the evaluation of breast FNAC specimens compared with conventional smears. Some advantages of LBC technique also have to be emphasized such as the easier way to collect the samples (overcoming poor cytological preparations) and the possibility for preserving material for ancillary tests such as immunohistochemistry and ISH methods. Therefore, LBC can be safely and routinely applied not only for the diagnosis, but also for the prognostic and predictive evaluation of primary and metastatic breast cancer.

Expert commentary

Based on its high diagnostic accuracy, LBC may be used as a valid alternative method to CC. Several papers underlined that LBC adoption reduces the number of non-diagnostic (both inadequate and artefactual) and indeterminate cases without impairing the distinctive malignant features of lesions. The storage of a variable amount of well-preserved cells allows for the application of immunocytochemical and molecular techniques that dramatically improve the efficacy of the morphologic diagnosis in each of the body site analyzed here. This assessment may promote continuous interest in the application of all the ancillary techniques with results as reliable as those obtained with small biopsies or surgical specimens.

Five-year view

The feasible results obtained with the application of LBC in different body lesions demonstrate the outstanding role of this method in achieving conclusive results with ancillary techniques. Relevant molecular testing features overlap and overrun those obtained with CC so that in future development, several guidelines may take into account the central role of LBC as an effective preparation for molecular testing and tailored therapies. This may be highly recommended in deeply located neoplasms and locally advanced patients who may benefit from any possible and available targeted therapies and longer survival supported by the cytological findings. Additionally, the 'nightmare' of a resistance mutational assessment may be foreseen on cytological samples establishing that cytology is a viable method for the use of molecular platforms and targeted NGS as a simultaneous testing for multiple mutations in the era of personalized targeted medicine.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Key issues

- Liquid-based cytology offers several advantages in the management and diagnostic evaluation of different body lesions based on a uniform, thin-layer distribution of cells.
- This technique introduces some cytomorphological features that are dissimilar from those of conventional cytology, including both architectural and cellular findings.
- Some authors demonstrated a higher statistical significant improvement in sensitivity and diagnostic accuracy after a 6-month period of training.
- Standardization in the method with uniform yields and homogenous cell distribution is one of the major strength attributed to this method.
- Liquid-based cytology residual material may be used for the application of ancillary techniques, including immunocytochemistry, molecular analysis and *in situ* hybridization with feasible and reliable results.
- The additional costs to the laboratory are counterbalanced by the decreased number of slides and the reduced slide surface.
- The application of molecular analysis with excellent results paves the way for the presurgical management of specific tailored therapies.
- Future research will provide great opportunities to analyze tumor genetics with a small quantity of cells and material as we have on cytology. This may offer new insights for the resistances to some specific therapies.

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