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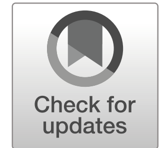
REVIEW AND PERSPECTIVES



Invited review—next-generation sequencing: a modern tool in cytopathology

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Abstract
In recent years, cytopathology has established itself as an independent diagnostic modality to guide clinical management in many different settings. The application of molecular techniques to cytological samples to identify prognostic and predictive biomarkers has played a crucial role in achieving this goal. While earlier studies have demonstrated that single biomarker testing is feasible on cytological samples, currently, this provides only limited and increasingly insufficient information in an era where an increasing number of biomarkers are required to guide patient care. More recently, multigene mutational assays, such as next-generation sequencing (NGS), have gained popularity because of their ability to provide genomic information on multiple genes. The cytopathologist plays a key role in ensuring success of NGS in cytological samples by influencing the pre-analytical steps, optimizing preparation types and adequacy requirement in terms of cellularity and tumor fraction, and ensuring optimal nucleic acid extraction for DNA input requirements. General principles of the role and potential of NGS in molecular cytopathology in the universal healthcare (UHC) European environment and examples of principal clinical applications were discussed in the workshop that took place at the 30th European Congress of Pathology in Bilbao, European Society of Pathology, whose content is here comprehensively described.

Keywords Molecular cytopathology · Next-generation sequencing · Fine-needle aspiration · Cell block · Direct smears · Liquid-based cytology

Introduction

Molecular cytopathology, namely the application of molecular techniques and genomic diagnostics to cytopathology, relies on the principle that, in addition to formalin-fixed paraffin embedded (FFPE) histologic material, cytology samples are also suitable for molecular testing [1]. Indeed, molecular cytopathology provides several advantages in many different settings [2]. The minimally invasive fine-needle aspiration (FNA) technique yields a tumor fraction usually higher than that of a biopsy specimen, which frequently contains abundant proportions of stromal and/or inflammatory cells [3]. In most

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routine cases, diagnostic cellular material is available on more than one cytopreparation, including direct smears, liquid-based cytology (LBC) and cell blocks (CBs) slides, which provides a wide variety of options for molecular testing by selecting the specimen preparation featuring the more pure population of neoplastic cells [3]. Since cytology samples are often collected in non-formalin-based fixatives, they offer the possibility of testing high-quality nucleic acids [4]. Moreover, smears enable to perform direct microscopic examination of the cell populations by rapid on-site evaluation (ROSE) to effectively triage for DNA/RNA extraction only those cases with adequate tumor fraction in order to avoid false-negative molecular results, which can commonly occur if the sample tested does not have a sufficient number or percentage of neoplastic cells [5].

The effective interplay between genomics and cytology can be exploited to address different clinical needs [6]. In particular, molecular cytopathology can help to predict patients' response to oncotherapy. Indeed, accurate and sensitive detection of actionable oncogenic mutations on cytological samples is now well established [7]. In addition, molecular cytopathology may refine microscopic diagnoses in challenging areas with a high level of diagnostic uncertainty and overlap [8]. In both predictive and diagnostic scenarios, single biomarker testing provides only limited and increasingly insufficient information; conversely, multigene mutational assays, such as next-generation sequencing (NGS), can simultaneously inform on multiple genes, requiring only a small amount of DNA [9, 10]. Several studies have described the feasibility and utility of NGS using cytological samples and have demonstrated sensitivities and specificities comparable to that of histological specimens [11], not only for DNA-based applications, but also to detect gene fusions of diagnostic or predictive relevance [12, 13]. Hence, the time is ripe to summarize the technical, clinical, and therapeutic aspects of NGS in molecular cytopathology. To this end, experts in this field discussed the various facets of this complex topic in a workshop that took place at the 30th European Congress of Pathology in Bilbao, whose content is here comprehensively described.

Pre-analytics and sample requirements

The cytopathologist' role

The adequacy of a cytological sample for molecular testing is influenced by a large number of variables. Some of them are beyond the cytopathologist's control, such as the skill and expertise of the radiologist/pulmonologist (trainee versus expert physicians). Similarly, tumor features such as the size, the site, and degree of fibrosis and of necrosis are also other independent variables. Nevertheless, the cytopathologist plays an important role in influencing these multiple pre-analytical steps, cumulatively referred to as pre-analytical procedures [2]. The cytopathologist selects the best quality cytology preparations for biomarker testing, having the responsibility to cancel the request for molecular assay whenever the cellularity is below the analytical requirements of the molecular assay [3]. Even minimal workflow changes can have broad implications [14]. As an example, the adequacy rate of CBs for NGS assays increases, when the process is optimized by concentrating all the material into a single block, avoiding refacing the CB and using mineral oil for de-paraffinization [15].

Due to this central role, the cytopathologist has an important and relevant role at the multidisciplinary team table. The tumor board is becoming more crowded with time [16]; in this setting, the cytopathologist can interact with interventional radiologists to ensure that adequate tissue amount is obtained, with surgeons to enquire whether a larger resection specimen is expected to be subsequently available for testing and with the oncologists to know whether the patient is a candidate for a targeted therapy and whether the oncologist's demand also include investigational gene targets required to enroll patients in clinical trials [2, 17].

In particular, several studies performed by the MD Anderson group have defined what parameters the cytopathologists should carefully evaluate when deeming sample adequacy for NGS analysis [3, 10, 11]. These can be roughly categorized as (i) specimen preparation type including collection media, fixative, and stain; (ii) specimen adequacy, i.e., cellularity and tumor fraction; (iii) nucleic acid extraction; and (iv) input DNA and assay requirements. Some general principles of cytological sample requirements for NGS are discussed below.

Cytological specimen preparation

The versatility of different cytological specimen preparations, including direct smears, cytospins, CB, and LBC offers a variety of options for NGS. FFPE CBs are the most widely utilized cytological substrate for NGS, in part due to their similarity to conventional histological tissue blocks. Most molecular laboratories that have validated NGS on histological FFPE tissue do not require separate validation for FFPE cytology CBs, except if processing techniques involve additional pre-analytic factors (such as alcohol fixation etc.). The relative ease of using CB sections frequently makes it the choice substrate for NGS, despite suboptimal nucleic acid quality from formalin fixation artifacts [2]. However, it should be borne in mind that the H&E section evaluated from the top of the CB does not necessarily represent the material deeper in the inclusion; therefore, the section following those adopted for testing should also be stained to ensure the adequacy of the sample. The need for additional separate validation and the medico-legal issues of sacrificing archival slides frequently

lead to an underutilization of cytologic smears for NGS analysis. However, direct smears provide high-quality nucleic acid, as well as the advantage of adequacy assessments by rapid on-site evaluation (ROSE) at the time of specimen acquisition and tumor enrichment via microdissection of whole cells. The updated Molecular Testing Guideline from the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors recommend that any cytology sample, with adequate cellularity and preservation, may be used for molecular testing [7]. This recommendation widens the use of cytology because the original recommendation preferred CBs over smears. This was based in systematic published reviews showing an excellent performance of smear preparations for molecular tests [2]. Recent studies have been also showing the use of NGS on cytological smears [2]. LBC has emerged to be a valid and alternative choice for NGS testing with increasing numbers of laboratories using LBC slides or residual LBC fluid for extracting nucleic acid. LBC has the advantage of direct smears in terms of high-quality DNA and using whole cells; however, it suffers from the inability to perform ROSE for tumor adequacy assessments and the need for additional pre-analytic validation for NGS assays.

Collection media, fixative, and stain

Optimization and standardization of pre-analytic variables are critical for any clinical NGS assay; this is especially true for cytological samples that utilize a variety of collection media, fixatives, and stains. Tissue from cytological aspirates can be collected in a multitude of media including saline, RPMI, formalin, and alcohol-based proprietary LBC media such as CytoLyt (Hologic, Bedford, MA) and CytoRich Red (Fisher Scientific, UK) that also serve as fixatives. Several studies have shown quantitative/qualitative differences in DNA yield and preservation between these various collection media [18]. Therefore, the need for rigorous and thorough validation with optimization of the various pre-analytic factors cannot be overemphasized. Likewise, both Diff-Quik and Papanicolaou stained slides have been shown to provide excellent results for NGS-based assays. A few studies have indicated that there may be quantitative differences in the DNA yield depending on the stain used [4, 18]; however, most institutions that perform NGS-based testing from previously stained smears utilize both Diff-Quik and Papanicolaou stains with comparable success.

Cytologic specimen adequacy assessment

The two main components to specimen adequacy assessment include evaluation of (i) overall cellularity and (ii) tumor cellularity. The overall cellularity which is defined by the number of nucleated cells in a sample is directly proportional to the DNA yield from a sample. Therefore, the higher the cellularity, the higher the DNA yield and the higher the chances of success of NGS [10]. However, the tumor cellularity (aka tumor fraction or tumor proportion) is critical for specimen adequacy assessment, as it relates to the analytic sensitivity of the testing platform [19]. Most NGS assays have a platform sensitivity of 5–10%, which requires a sample to have a minimum tumor cellularity of 10–20% to reliably detect a mutant allele (present in tumor cells only) from the background of wild-type alleles. Low tumor fraction samples therefore have a higher chance to false-negative results if it fails to meet the minimum threshold for platform sensitivity [2]. Tumor fraction can frequently be enhanced by tumor enrichment techniques, albeit at the cost of overall cellularity of the sample [3].

Nucleic acid extraction

Several studies have outlined nucleic acid extraction from cytological samples for NGS analysis. These include scraping or cell-lifting of tumor cells directly off smears, cytospins, and LBC slides or from unstained sections of cell blocks by matching a corresponding H&E stained slide and/or direct extraction from collection media such as residual LBC and post-centrifuged FNA supernatants. The limitation of nucleic acid extraction directly from collection media is the inability to assess for tumor fraction which may lead to potential false-negative results when tumor DNA is not included in the sample. However, preliminary studies have shown promising results as these substrates frequently provide higher quantity/quality tumor DNA for NGS analysis [15, 20]. Methods of tissue extraction as well as the type of glass slide used for smear preparation can have varying impact on the nucleic acid retrieval and DNA yield. For example, tissue extracted by cell scraping has higher DNA yield than those extracted by cell-lifting using the PinPoint solution [9]. Further, fully frosted glass slides tend to yield significantly less amounts of DNA than non-frosted or positively charged glass slides [9]. Therefore, optimizing pre-analytic variables for nucleic acid extraction is needed to best utilize cytology samples for NGS assays.

Input DNA and assay requirements

A minimum tumor cell proportion is needed for NGS (around 10 to 20%). In case of a lower number, there would be a chance to miss mutations due to dilution with DNA from benign cells. Macro- or laser capture microdissection can easily be performed in order to enrich for tumor cells. Although even minimal amounts (< 10 ng) of input DNA obtained from

selected cells may be sufficient, the microdissection procedure may lead to suboptimal DNA quality.

The DNA requirements for NGS assays depend on the platform used for testing. The most common NGS platforms that have been used for cytological samples are the bench-top sequencers from Ion Torrent (ThermoFisher Scientific, Waltham, MA) and Illumina (Illumina, San Diego, CA). The sequencing chemistries of the two platforms are different: Ion Torrent uses an amplicon-based NGS assay, while Illumina was generally associated with a hybrid capture–based assay. The manufacturer recommended input DNA requirements also vary for the two platforms with Ion Torrent recommending 10 ng and Illumina recommending at least 50 ng [17]. Both sequencing assays have been shown to be compatible with cytological samples, although Ion Torrent has an inherent advantage with small volume cytology samples due to its lower input requirement.

Validation

Although NGS is a fascinating technique, as with any clinical assay, specific validation to optimize pre-analytic cytological variables in individual laboratories prior to use for patient care is absolutely critical. As an example, the higher quality DNA extracted from cytological slides may allow for a lower input threshold for NGS assays than that suggested by the manufacturer for FFPE specimens [10]. The validation of NGS, including pre-analytical, nucleic acid preparation, sequencing, and bioinformatics steps, should yield parameters of analytical sensitivity, specificity, accuracy, and precision. Depth of coverage, average, and uniformity of coverage should also be determined during the validation process as well as validating for minimum sequence coverage for the main genetic alterations of diagnostic interest. A high depth of coverage is especially important when the DNA input and the percentage of malignant cells are scant. While in earlier studies, a minimum coverage of 500× with at least a 10% mutant allele frequency was generally used as cutoff for a variant to be considered true, more recently, in the routine diagnostic, a minimum coverage of 200× with at least a 5% mutant allele frequency is regularly used [21]. It is impossible, however, to validate any single gene variant, even when a large retrospective collection of routine cytological specimens, homogeneous for the source, type, fixation, staining, and tumor cell enrichment modalities, with a known mutational status for all clinically relevant genes, is available. As it will be discussed later, commercial FFPE multiplex reference standards can represent a solution, at least when validating NGS assays on CBs.

Implementation of NGS in clinical practice

NGS in a universal healthcare systems

Beyond pre-analytical and technical factors, the role and potential of NGS in molecular cytopathology is also strongly influenced by more general considerations. In particular, it is very important to consider the type of healthcare systems in which NGS technology is employed. Indeed, several differences may occur between countries adopting well-resourced, reimbursement-based systems and those with the universal healthcare (UHC) organization [22]. In fact, in a private setting, insurance coverage can ensure the repayment of extensive tumor sequencing; thus, the NGS technology is fully exploited as a "one-stop-shop" for all possible genomic targets. Conversely, in a UHC organization, resources are finite and the main efforts are spent to provide at least the molecular information that can directly guide standard-of-care management. From an economic point of view, the larger the number of hospitals carrying out molecular tests is, often on a limited number of samples, the more it costs raise. In fact, resources are needed to afford NGS platforms, expert laboratory team, bioinformatics infrastructure, and data storage facilities. Furthermore, another important requirement for a center performing broad NGS molecular diagnostics is the need to develop a dedicated molecular tumor board (MTB) to really make effective in clinical practice genetics-guided cancer care [23]. Mutations should be carefully annotated and classified as part of (i) treatment recommendations, (ii) eligibility for experimental treatments, and (iii) patients without any therapeutic option [22]. Ideally, a centralized database for annotation and curation of reports would be cost-effective, allowing standard reports with clear recommendations to be provided to clinicians. As an example, the German Network for Genomic Medicine (NGM) in lung cancer is a healthcare network providing NGS-based multiplex genotyping for all inoperable lung cancer patients [24]. However, more efforts have to be spent to implement in clinical practice MTB, as a recent survey in the Netherlands showed that only 5% of non-academic hospitals had access to a MTB [23].

Gene panels

The choice of a gene panel is one of the main factors that strongly influence the NGS use [25]. There are a number of possible gene panel designs, including small panels covering the hotspots regions of the most common actionable genes (covering up to 10–15 genes). Intermediate-size panels (consisting of up to 50 genes) allow oncologists to enroll patients in clinical trials. Currently, larger sequencing panels are being intensively employed for tumor mutational burden

(TMB) assessment, with little degree of concordance on the threshold to define cases with high TMB [26–28]. As a general rule, while larger cancer care comprehensive centers may be more interested in larger NGS analysis to evaluate a large number of biomarkers, small panels seems to fill in an intermediate space between large panels and PCR-based assays, underlining the concept that NGS is a versatile technology, whose aim and scopes greatly differ among institutions and clinical setting [26, 29]. As a matter of the fact, small gene panels require a lesser abundant DNA input, which makes NGS analysis feasible even on small tissue samples, including cytology [30]. As an example, the narrow NGS panel (SiRe®) developed at the University of Naples Federico II covers 568 clinical relevant mutations in six genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *KIT*, and *PDGFRa*) involved in NSCLC, gastrointestinal stromal tumor, colorectal cancer (CRC), and melanoma [29, 31, 32]. In a recent investigation on NSCLC routine samples, out of a total of 322 cases, only 28 (8.7%) failed to produce an adequate library. The performance of SiRe® Panel was more than satisfactory showing an average of 166,206.91 reads per sample with a median read length of 130.64 bp. In addition, the NGS workflow allowed a very cost-effective batching of samples (16 per run on 316v2 chip, ThermoFisher Scientific), regardless of the type of tumor and the pathological preparations. As a result, turnaround time (TAT) can be as short as recommended by international guidelines.

Consistency and reproducibility of NGS testing on cytological samples

Although there is a consensus that NGS can be carried out on archival smears, most of the available data reflect only single institutions rather that multicenter studies for inter-laboratory comparison of protocols. Although it is undeniable that surplus routine patient smears are the best option for validating gene mutational analysis, studies on cytological smears are difficult because smear slides are not reproducible or replaceable. However, the molecular cytopathology community can share experience and face new challenges on artificial cytological slides [33]. In particular, this opportunity has been seized thanks to the Molecular Cytopathology Meeting Group, which meets annually in Naples with a goal of developing collaborative projects across the globe. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) 9 is a robust technology that can be used to introduce in cell lines a range of variants, including single-point mutations and insertion-deletion mutation, assessing NGS work library complexity, required depth of coverage, mutant allele frequencies (AFs), and overall performance.

NGS testing on lung cancer cytological specimens

The European Congress of Pathology cytology workshop also stated that the most common application of NGS panel in cytology is for predictive biomarker testing in lung cancer [34]. This is due to the fact that a large fraction of NSCLC patients are diagnosed in advance, inoperable stage of disease and cytology is commonly the only available tumor material. According to recent guidelines [7, 35], it is a standard of care to test all advanced-stage patients with non-squamous morphology at least for *EGFR* (indel-mutations), *BRAF* (mutations), *ALK* (gene fusions and mutations), and *ROS1* (gene fusions). *MET* (mutations and amplification), *RET* (gene fusions), *NTRK1/2/3* (gene fusions), and *ERBB2* (mutations and amplification) should be included in any expanded panel, in case there is access to the corresponding targeted drugs via clinical trials or compassionate use programs. NGS assays can be reliably carried out on any kind of routine lung cancer cytology specimen to assess DNA targets. In fact, in the experience of one of the authors, the rejection rate for NGS-based predictive gene mutation testing in cytology using laser microdissection needed is less than 5%, even in pauci-cellular specimens as bronchial secretions, for example. However, publications are still limited for simultaneous RNA-based detection of gene rearrangements [13]. As a general rule, there are two major approaches used for detection of gene fusions in lung cancer by NGS, either to sequence DNA using hybrid capture method or to sequence RNA (cDNA) by amplification-based methods [25]. This latter approach requires little RNA input but may fall short to capture large exons/introns sequences that are most frequently involved in the fusion of interest in lung cancer [25]. Future perspectives may lead to the validation of hybrid capture strategies; as an example, the Memorial Sloan Kettering Cancer Center Experience suggests that broader-based NGS assays may be performed even on cytological samples when the whole testing workflow is optimized by a cohesive group-based approach including clinical, cytopathology, surgical pathology, molecular, and bioinformatics teams [15]. Moreover, technology is advancing at a rapid speed, and novel transcriptome-based platforms are emerging. In particular, when the extracted RNA is of poor quality and the target capture amplification fails, the alternative nCounter Analysis System (NanoString Technologies Inc., Seattle, WA) can be a viable choice to provide a single-tube test for *ALK*, *ROS1*, and *RET*. [36, 37]

NGS testing to refine uncertain pancreatic cytological diagnoses

The workshop at the 30th European Congress of Pathology also aimed to illustrate the clinical potential of NGS on

cytology. To give a hint of the possibility of this modern technology to refine uncertain cytological diagnoses, the paradigm of FNA of pancreatic lesions was used. Pancreatic tumors are a heterogeneous group of lesions, both cystic and solid, malignant and benign, or of low malignant potential, which need to be distinguished from mimickers (chronic pancreatitis and pseudocysts). There is a good phenotype-genotype correlation. Pancreatic ductal adenocarcinoma (PDAC) has a high prevalence of *KRAS*, *CDKN2A/p16*, *DPC4/SMAD4*, and *TP53* mutations, which are relatively uncommon in other forms of pancreatic tumors. Pancreatic acinic adenocarcinoma (PAAC) features *APC* inactivation (by promoter hypermethylation or gene mutation), or less frequently mutations of the beta-catenin gene *CTNNB1* (like *APC* an element of the *WNT* pathway). *CTNNB1* mutations characterize instead of solid pseudopapillary neoplasms (SPNs), where they are found in the vast majority of cases. Among cystic tumors, intraductal papillary mucinous neoplasms (IPMNs) typically have *GNAS*, *RNF43*, and *KRAS* mutations, the latter also common in mucinous cystic neoplasms (MCNs), while serous cystadenoma (SCA) is characterized by *VHL* alterations (inactivating mutation or loss of heterozygosity in sporadic cases, germline mutations in patients with the Von Hippel-Lindau syndrome). Sporadic pancreatic neuroendocrine tumors (Pan-NETs) have mutually exclusive *DAXX* and *ATRX* mutations and *MEN1* mutations, while *MEN1*, *VHL*, NF-1, or *TSC1/2* germline mutations are found in Pan-NETs that develop in patients with inherited neuroendocrine genetic syndromes [38, 39].

Surgical resections of pancreatic tumors are often complex procedures mandating a preoperative diagnosis as accurate as possible. Even for unresectable tumors, an accurate diagnosis is essential to define the best treatment options [40, 41]. Accuracy has greatly improved since the widespread introduction of endoscopic ultrasound (EUS)–guided FNA. However, in many cases, the preoperative evaluation remains inconclusive because of insufficient material or limited cellularity, leading to atypical/suspicious cytopathologic diagnoses. Single gene, typically *KRAS*, analysis of cytologic material has proven useful, but NGS is opening new avenues to improve the preoperative diagnosis [42–44]. NGS has three features that make it a suitable adjunct to the preoperative evaluation of pancreatic lesions: (a) it has high analytical sensitivity, essential to characterize those samples that are limited or heterogeneous; (b) it allows for multiple gene testing; (c) it allows for relative quantification of the mutant allele in the specimen (mutant allele frequency, MAF).

In the last 10 years, NGS has been successfully applied to the evaluation of (i) solid masses [45–55]; (ii) cyst fluid [45, 47, 53, 56–61]; (iii) other fluid material (e.g., pancreatic juice, peripheral blood) [62–64]. The results of these studies have consistently shown a great promise for the preoperative classification of both solid lesions and cysts. While *KRAS* remains the gene most widely studied for pre-operative single gene tests, NGS analysis of multiple gene markers (to include *KRAS*, *CDKN2A/p16*, *DPC4/SMAD4*, *TP53*, *GNAS*, *RNF43*, *CTNNB1*, *BRAF*, *PIK3CA*) is becoming very attractive for the diagnosis, management and pre-operative risk stratification of patients with pancreatic cancer [44, 55, 58].

Others application of NGS on cytological specimens

There has been a lot of effort in developing molecular tests, which could improve the sensitivity and specificity of thyroid FNA in order to reduce the need for surgery. The recently developed ThyroSeq NGS assay, designed for sensitive detection of thyroid cancer-related gene mutations and rearrangements on FNA specimens, shows promising results. However, it is only available as a commercial test, outsourced in a private US laboratory [65, 66]. A generic cancer panel, such as Ion AmpliSeq Cancer Hotspot Panel v2 (CHPv2), which includes the genes most frequently mutated in papillary thyroid carcinomas, is commercially available and may represent an alternative to thyroid-specific panels [67].

Effusions are an example of a very rich cytological material where we can study molecular alterations during cancer progression. Most of the applications of NGS in effusions are related to in lung cancer, but also in other cancer types as for example in ovarian cancer for *BRCA* testing [68]. In salivary gland, pancreas, and biliary tract cytology, where the morphology can be very challenging, NGS can add to an unequivocal diagnosis [50]. In pancreas and biliary tract cytology, the amount of tumor cells may not be abundant, but since around 200 cells are enough to perform NGS, it will be applicable to most cases.

Conclusion

Since morphological evaluation of neoplastic cells aided by immunocytochemical studies commonly provides only limited information on diagnostic, prognostic, and predictive tumor features, molecular techniques have deeply transformed the way in which we practice cytopathology. In this setting, NGS is a modern tool for modern cytopathologists. It is widely held that this technique is feasible on most cytological samples. Future challenges can give tremendous opportunities to fully exploit the potential of this fascinating technique. In particular, we foresee that a new generation of sequencing platforms may better capitalize the informativeness of pauci-cellular cytological samples. Moreover, larger gene panels may provide information not only on the most frequent and clinical validated targets, but also on a growing number of actionable molecular modifications. Modern cytopathologists should more and more engage with the clinical, molecular, and therapeutic aspects of cancer care to play a significant role in personalized medicine.

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Compliance with ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this review, formal consent is not required.

Conflict of interest The authors declare that they have no conflict of interest.

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