





REVIEW

Next generation sequencing in cytology

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Abstract

The application of next generation sequencing (NGS) technology to cytological samples has significantly modified molecular cytopathology practice. Cytological samples represent a valid source of high-quality DNA for NGS analysis, especially for predicting patients' response to targeted treatments and for refining the risk of malignancy in indeterminate cytological diagnoses. However, several pre-analytical factors may influence the reliability of NGS clinical analysis. Here, we briefly review the challenges of NGS in cytology practice, focusing on those pre-analytical factors that may negatively affect NGS success rates and routine diagnostic applications. Finally, we address the future directions of the field.

KEYWORDS

biomarkers, cytopathology, FNA, molecular cytopathology, next generation sequencing

1 | INTRODUCTION

In today's rapidly evolving field of molecular cytopathology, modern cytopathologists play a key role in bridging the gap between conventional microscopy and novel molecular technologies.¹ The advent of targeted and personalised therapies has completely modified the way advanced cancer patients are managed.² As opposed to standard therapeutic regimens (eg, radio-chemotherapy), these novel drugs have proven highly effective in dramatically improving the overall clinical outcomes of advanced cancer patients while reducing unwanted severe adverse events and toxicities. Despite these remarkable advantages, the administration of these therapies is strictly dependent on the identification of specific molecular "targets."³ Nowadays, several biomarkers have already been approved as predictors of response to targeted treatments while other promising ones are currently under investigation.² Because surgical biopsies in advanced cancer patients often require impracticable

and invasive procedures, cytological samples frequently represent the only available tissue material for morph-molecular purposes.⁴⁻⁶ Besides its predictive role, cytological sampling is also a useful diagnostic approach for superficial or deep-seated nodules.⁷ In this setting, nucleic acids extracted from cytological samples may be exploited to refine the malignancy risk of cases classified as "atypical" or as "of undetermined significance."⁸ However, a number of technical hurdles must be overcome before cytological samples can be widely adopted clinically for molecular purposes. For instance, a common setback is that despite providing high-quality nucleic acids, cytological samples commonly yield only limited nucleic acid input compared with histological specimens.⁹

The introduction of next generation sequencing (NGS) technologies has been instrumental in overcoming this limitation.^{10,11} Indeed, NGS is a fascinating tool that enables cytopathologists to analyse different biomarkers simultaneously, even when the starting material features a low tumour content.¹⁰⁻¹² In particular, NGS

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technologies are based on three main sequencing approaches: by synthesis, by hybridisation, and by ligation.¹³ In spite of these differences, all three approaches employ the same exact four-step workflow: (a) library generation; (b) clonal amplification of the single generated fragments; (c) massive parallel sequencing, and (d) data analysis.¹⁰ Currently, because of its affordable cost and time-saving protocols, many molecular laboratories around the world are implementing NGS platforms in their routine practice. However, despite its popularity, a number of different pre-analytical factors seem to compromise NGS success rates on cytological specimens. For instance, although cytological samples are generally suitable for NGS analysis (Table 1),^{14,15} care must be taken to validate protocols developed for non-formalin-fixed and paraffin-embedded (FFPE) specimens, such as smears and liquid-based cytological (LBC) samples.¹⁶

In this review, we briefly describe the current role of NGS in cytology particularly by focusing on critical pre-analytical factors and routine diagnostic applications, and address the future directions of the field.

2 | PRE-ANALYTICAL VARIABLES IN CYTOLOGY

One of the most common pre-analytical factors capable of negatively affecting NGS results is the fixative adopted. In particular, smears and LBC samples feature the remarkable advantage of providing better nucleic acid quality than FFPE samples, such as cell blocks (CBs).⁹ The problem with CBs though is that fixation in formalin, particularly if prolonged over time, may give rise to C > T sequence artifacts.^{17,18} Among non-formalin fixation modalities, whereas some studies have reported that air-dried and Diff Quik-stained smears yield higher quality nucleic acids than ethanol-fixed and Papanicolaou-stained smears,¹⁹ others have reported divergent evidence.²⁰ In this setting, despite these differences, fixation and staining modalities are not likely to hamper accurate NGS analysis.²¹

In the case of LBC preparation, as expected, a higher nucleic acid yield and quality has been reported when alcohol-based fixatives, such as CytoLyt (Hologic), have been adopted as opposed to formaldehyde-based ones, including CytoRich Red (Thermo Fisher Scientific).^{9,21}

In addition, cytopathologists try to avoid sacrificing the morphology of non-replicable diagnostic slides for molecular analysis.⁹ Thus, a preliminary evaluation of the most relevant factors that may affect NGS success rates of cytological specimens is crucial. In this regard, cytopathologists should review the pathological material on the slides to select the best high-quality smears or representative CB sections. In particular, careful attention should be paid to select slides displaying the highest tumour cell content while simultaneously avoiding, if possible, any contaminant that may interfere with the NGS analysis.^{9,21} This procedure is carried out for each selected slide, with the selection of the tumour-enriched areas.^{9,21} At this point, the cytopathologist should evaluate whether the tumour cell content of the sample can satisfy the analytical sensitivity of the molecular assay employed.^{9,21} To this end, despite the lack of a universally accepted cut-off for NGS analysis, it has been proposed that the tumour fraction should be more than twice the limit of detection recommended by the assay.²²

On the other hand, when samples fail because of low cellularity, additional CB sections or smears can be used.^{9,21} However, when choosing among different cytopreparations, cytologists should bear in mind the distinct advantages and disadvantages that each one entails. For example, both direct smears and LBC specimens have an advantage over CB types because they contain whole cells and whole nuclei from which to extract high-quality nucleic acids. However, only direct smears are suitable for rapid on-site evaluation (ROSE), a technique that enables cytopathologists to better manage the aspirated material for morph-molecular purposes.²³ The downside of direct smear preparations is that the number of available slides is very limited. This may lead to the sacrifice of valuable diagnostic morphological material and require a careful time-consuming

TABLE 1 Different cytological preparations: Pros and cons

Preparation	Pros	Cons
Direct smear	High-quality nucleic acids Possibility to perform ROSE, useful to triage the aspirated material	Necessity of careful additional validation steps for any given molecular approach Unique and unrepeatable
CB	Possibility to perform ancillary studies without the need of additional validation Ensure the preservation of diagnostic slides	Low quality nucleic acids due to formalin fixation Impossibility to perform ROSE
LBC	Avoid inadequate administration of the aspirated material by untrained physicians Aspirated material can be rapidly collected and preserved in alcohol-based media	Impossibility to perform ROSE Variable yield and quality of nucleic acids depending on the fixative adopted
Supernatant	High yield and quality of nucleic acids Ensure the preservation of diagnostic material Enabling molecular analyses even when cytological slides are inadequate or insufficient	Impossibility to perform ROSE Impossibility to perform morphological evaluation

Abbreviations: CB, cell block; LBC, liquid-based cytology; ROSE, rapid on-site evaluation.

TABLE 2 Principal pre-analytical factors that may affect next generation sequencing analysis on cytological samples and clinical applications

Pre-analytical factors	Fixation modalities Sample preparation Staining Assessment of neoplastic cell content Type of adopted slide Extraction techniques
Clinical applications	Diagnosis Refine the risk of malignancy Therapy

validation step before NGS routine application.^{9,21} Conversely, CBs do not require additional validation steps and are a valuable option for storing tissue material. However, like other FFPE samples, their quality may be compromised by formalin fixation artifacts.^{9,17,21,24} (Table 2).

3 | CLINICAL APPLICATION OF NGS TO CYTOLOGICAL SAMPLES

The success of NGS testing in cytological samples from advanced cancer patients has been widely demonstrated.²¹ For instance, corroborating evidence has highlighted the possibility of assessing clinically relevant predictive biomarkers in non-small cell lung cancer (NSCLC) patients by applying NGS to different cytological preparations. Indeed, the guidelines of the first edition of the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology recommended the use of CB samples over other types of cytological preparations for advanced-stage NSCLC molecular testing.⁵ Instead, the guidelines reported in the subsequent updated version point out the usefulness of smears for molecular purposes.⁶

In a recent study, we described and validated a newly developed narrow NGS panel, which we called SiRe[®]. This panel, which was designed to cover 568 clinically relevant mutations in six different genes, enabled us to analyse a total of 164 (91.1%) out of 180 routine NSCLC cytological samples, showing an 18.3% rate of mutated cases for Epidermal Growth Factor Receptor (*EGFR*), 28.0% for Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*), 0.6% for Neuroblastoma RAS Viral Oncogene Homolog (*NRAS*), and for 4.9% V-Raf Murine Sarcoma Viral Oncogene Homolog B (*BRAF*).²⁵ A similar NGS success rate was reported by Zhang et al. Interestingly, in this limited experience, as it was performed on only 16 CBs derived from lung adenocarcinomas (pleural effusions or fine needle aspiration [FNA]), NGS succeeded in analysing almost all samples (93.8%, 15/16).²⁵ In particular, it detected nine *EGFR* mutations, one Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*) mutation, and one *KRAS* mutation.²⁶ Likewise, the suitability of non-formalin-fixed smears for NGS analysis was demonstrated by Velizheva et al.¹⁵ Comparing NGS results with those obtained on matching FFPE

histological and CB specimens, Velizheva and colleagues reported an overall success rate of 100.0% for DNA analysis and 92.0% for RNA analysis on stained smears. Interestingly, the smears showed a high sensitivity (100.0% and 100.0%) and specificity (96.0 and 100.0%) for both DNA and RNA analysis, respectively.¹⁵ In line with these results, Karnes et al²⁷ reported that despite the lower DNA input, cytological samples (both Diff Quik- and Papanicolaou-stained smears) and matching FFPE histological specimens featured similar results in terms of sequencing run parameters and single nucleotide variants detection (overall concordance of 99.5%).

In addition to direct smears and CBs, LBC may also play a crucial role in biomarker assessment for predictive purposes in advanced-stage NSCLC patients. For example, Reynolds et al²⁸ evaluated the possibility of adopting archived residual cell pellets from LBC preparations as a reliable starting material for NGS analysis. Overall, 20 archived LBC cell pellet samples were retrieved and underwent NGS analysis. Focusing their attention on *EGFR* mutations, 12 mutated cases were reported. Of note, in all instances the *EGFR* mutations were further confirmed by real-time polymerase chain reaction.

As we mentioned above, NGS analysis in cytological samples may be a valid tool not only to predict treatment response to targeted treatments in advanced cancer patients but also to refine the risk of malignancy in cases classified as “atypical” or as “of undetermined significance.”⁸ A few years ago, we reported our experience with an NGS-targeted panel of 50 genes. Remarkably, the NGS panel was successfully applied to 34 (91.8%) out of 37 Diff Quik-stained smears, including indeterminate cases retrospectively retrieved from our routine thyroid FNA practice. Overall, 22 (64.7%) out of 34 analysed samples harboured *BRAF*, *NRAS*, or Rearranged During Transfection (*RET*) alterations. Interestingly, the NGS approach demonstrated high sensitivity (89.4%), specificity (85.7%), and accuracy (88.4%) rates.²⁹ Consistently, Le Mercier et al used the same 50-gene NGS panel on CBs and stained smears from thyroid FNAs to evaluate the risk of malignancy in patients with morphologically indeterminate diagnosis. As in the previous studies, they reported that NGS successfully identified gene mutations strictly associated with thyroid cancer development (eg, *BRAF*, *NRAS*, *KRAS*, and Phosphatase And Tensin Homolog [*PTEN*]), thus suggesting that NGS may increase the sensitivity of FNA diagnosis of indeterminate lesions.³⁰ The analytical performance of NGS on FNA samples was further confirmed by Nikiforov et al. Using the ThyroSeq v2.1 panel to analyse a large series of 465 consecutive thyroid FNAs with indeterminate diagnosis, they detected 6.7% mutated cases. Interestingly, NGS was able to adequately assess a high risk of malignancy in almost all (20/22) nodules with indeterminate cytological diagnosis. In particular, NGS showed an overall accuracy of 91.8%, a sensitivity of 90.9%, a specificity of 92.1%, a positive predictive value of 76.9%, and a negative predictive value of 97.2%.³¹ In a subsequent experience the same group evaluated the analytical performance of a new version of the ThyroSeq panel, the ThyroSeq v3, an NGS DNA- and RNA-based panel able to cover 112 gene alterations and to distinguish malignant from benign lesions. For the evaluation, they applied ThyroSeq v3 to 413 specimens (including 175 thyroid FNA samples with indeterminate diagnosis) and to matching

TABLE 3 Summary of the studies that adopted next generation sequencing on cytological samples

Platform	Panel	Sample type	Number of analysed samples	Adequate sample rate	Clinical application	Reference
Ion S5 System™ (Thermo Fisher Scientific)	Custom Panel (7 genes)	Direct smear, Cell block	180	91.1%	Therapy	25
Ion PGM™ (Thermo Fisher Scientific)	NextDaySeq Lung panel (7 genes)	Cell block	16	93.8%	Therapy	26
Ion PGM™ (Thermo Fisher Scientific)	Oncomine DNA panel for Solid Tumors and Fusion Transcripts (26 genes)	Direct smear	8	92.0% (RNA-based) 100.0 (DNA-based)	Therapy	15
HiSeq 2000 (Illumina)	WU-CaMP27 panel (27 genes)	Direct smear	5	100.0%	Therapy	27
Ion PGM™ (Thermo Fisher Scientific)	Ion AmpliSeq Cancer Hotspot Panel (50 genes)	Liquid-based cytology	49	77.5%	Therapy	28
Ion PGM™ (Thermo Fisher Scientific)	Ion AmpliSeq Cancer Hotspot Panel (50 genes)	Direct smear	37	91.8%	Refine the risk of malignancy	29
Ion PGM™ (Thermo Fisher Scientific)	Ion AmpliSeq Cancer Hotspot Panel (50 genes)	Direct smear, Cell block	34	85.2%	Refine the risk of malignancy	30
Ion Torrent PGM or Ion Proton (Thermo Fisher Scientific)	ThyroSeq v2.1 panel (56 genes)	Supernatant	465	100.0%	Refine the risk of malignancy	31
Ion Proton (Thermo Fisher Scientific)	ThyroSeq v3 panel (112 genes)	Supernatant	175	100.0%	Refine the risk of malignancy	32
Next-Seq500 (Illumina)	AmpliSeq Comprehensive Panelv3 (161 genes)	Supernatant	33	97.0%	Diagnosis	34
iSeq platform (Illumina)	AmpliSeq for Illumina Cancer Hotspot Panel v2 (50 genes)	Direct smear	32	100.0%	Refine the risk of malignancy	35
Ion S5 System™ (Thermo Fisher Scientific)	Oncomine BRCA Research Assay (2 genes)	Direct smear	11	100.0%	Therapy	36
Ion Proton (Thermo Fisher Scientific)	Ion AmpliSeq Cancer Hotspot Panel v2 (50 genes)	Supernatant, Cell block	35	100.0%	Diagnosis, Refine the risk of malignancy, Therapy	38
Ion PGM™ (Thermo Fisher Scientific)	Solid Tumor Focus Assay (69 genes)	Supernatant	30	100.0%	Therapy	39
Ion Proton (Thermo Fisher Scientific)	Ion AmpliSeq Cancer Hotspot Panel v2 (50 genes)	Supernatant	116	89.7%	Therapy	40
NextSeq (Illumina); digital droplet PCR	IAseq Targeted ActionableSolid Tumor Panel (20 genes)	Supernatant	17	100.0%	Therapy	41
Ion Proton (Thermo Fisher Scientific)	Ion AmpliSeq Cancer Hotspot Panel v2 (50 genes)	Supernatant	156	83.0%	Refine the risk of malignancy	42

histological surgical resections. Significantly, the panel showed an overall accuracy of 90.9%, a sensitivity of 98.0%, and a specificity of 81.8% compared with the histological samples.³²

Besides lung cancer and thyroid neoplasms, NGS technology may also be applied to other types of cytological samples. For instance, Harris et al³³ showed that NGS is a valid ancillary approach for atypical urine cytology samples and a potential screening tool for low-grade urothelial carcinomas, which are commonly missed by

both cytology and cystoscopy approaches. Similarly, Carrara et al³⁴ demonstrated that the application of NGS to pancreatic FNAs for the identification of *KRAS* alterations may be a valid complementary diagnostic strategy to traditional morphological and ancillary approaches for the diagnosis of pancreatic ductal adenocarcinomas. Further, Yamamoto et al demonstrated that NGS can be useful in evaluating the malignant potential in salivary gland tumour cytological specimens. Indeed, NGS yielded a sensitivity and a specificity

of 71% and 94%, respectively. Notably, only 4/14 malignant cases showed no alterations, whereas only 1/18 benign lesions showed a mutation.³⁵ Fumagalli et al highlighted the suitability of NGS for breast cancer (*BRCA*) 1/2 gene analysis on cytological samples derived from neoplastic ascites of ovarian cancer patients. In this study, NGS of cytological samples showed a success rate of 100.0%. Indeed, complete concordance was seen between cytological and histological analyses. In particular, two cases were wild types, and nine cases harboured *BRCA* 1/2 somatic or germline alterations.³⁶ The results are summarised in Table 3.

4 | SALVAGING THE SUPERNATANT

Preserving irreplaceable and irreproducible diagnostic cytological specimens still remains a major issue for molecular analysis. One possible solution to this pitfall is to exploit the nucleic acids from supernatant fluids, primarily nucleic acid residues from FNA needle rinses or those obtained after cell pelleting and centrifugation during cytological specimen preparations.³⁷ Moreover, the nucleic acids recovered from supernatant fluids could also be harnessed when diagnostic cytology slides are inadequate or insufficient for molecular purposes.³⁷ In fact, in these cases, despite the evidence of diagnostic tumour cells for morphological purposes, the extremely low tumour cell content in terms of percentage of neoplastic cells may not be suitable for NGS analysis.

Several studies have been conducted to validate the feasibility of exploiting nucleic acids extracted from supernatant fluids for NGS analysis. A couple of years ago Roy-Chowdhury et al performed NGS analysis on DNA extracted from post-centrifuged supernatants from FNA needle rinses collected in an RPMI medium. They were able to analyse 13 (54.2%) out of 24 malignant cases featuring a limited volume ($n = 6$) or inadequate cytological tissue material ($n = 7$) for molecular purposes on tissue cytological samples.³⁸ A similar line of research was followed a year later by Janaki et al.³⁹ To further validate the idea of using discarded specimen fluids for molecular analysis, they compared the molecular results of NGS from tissue samples with those from supernatants in 30 endobronchial FNAs. Interestingly, they reported a complete concordance rate (100.0%) between the two groups. Hannigan et al confirmed the practicality of applying NGS analysis to supernatant-derived DNA for predictive purposes. Overall, they detected somatic mutations in the vast majority (81.7%) of the analysed samples. Interestingly, half of the patients harboured a druggable mutation. They also obtained concordance rates of 100.0% and 96.0% between the mutation identified in the supernatants and that found in the matching FNAs or core needle biopsies.⁴⁰ Likewise, Guibert et al adopted the NGS approach to analyse DNA extracted from FNA supernatants of 12 lung adenocarcinomas. Of note, they obtained a perfect concordance rate between FNA-tissue derived DNA and supernatant-derived DNA obtained from newly diagnosed patients and those with adenocarcinomas who became resistant to tyrosine kinase inhibitors (TKIs).⁴¹ Finally, Ye et al recently adopted the same strategy by applying NGS

analysis to DNA extracted from supernatants of FNA of thyroid nodules. Remarkably, they were able to improve the diagnostic accuracy in indeterminate nodules. Indeed, they found that two (6.5%) out of 31 cases with indeterminate cytological diagnosis harboured *BRAF* exon 15 p.V600E, a somatic mutation highly associated with papillary thyroid carcinoma.⁴² Among these two cases only one had a histological follow-up, featuring a papillary thyroid carcinoma diagnosis, further confirming the key role of molecular testing in cases with indeterminate cytological diagnosis. The results are summarised in Table 2.

5 | QUALITY CONTROL IN CYTOLOGY

Although cytological samples provide a higher quality of nucleic acids than histological samples, air-dried or ethanol-fixed cytological smears usually require a careful validation process before being considered suitable for NGS analysis in routine clinical practice.¹⁴⁻¹⁶ In addition, owing to their unique and unrepeatable nature, smears are generally not suitable for quality control studies that evaluate the consistency and reproducibility of NGS results among different laboratories. Thus, to circumvent loss of specimen, LBC preparations may play a relevant role in generating quality controls that may be distributed across different laboratories. For this reason, the international Molecular Cytopathology Meeting Group, a consortium composed of laboratories with very high expertise in molecular analysis of cytological samples, has designed, developed, and validated artificial genomic reference standards in cytocentrifuge/cytospin format.^{43,44} In brief, in the "first round" of this international ring trial, cell lines were engineered to harbour mutations in *EGFR* exon 19 p.E746_A750del, *KRAS* exon 2 p.G12D, *NRAS* exon 3 p.Q61L, *BRAF* exon 15 p.V600E, and *PIK3CA* exon 20 p.H1047R at different dilution points (10%, 5%, and 1% allelic frequency, and a wild type control).⁴³ Slides, containing 2×10^6 cells, were distributed to each laboratory. Overall, all laboratories adopting NGS were able to detect all the engineered mutations at an allele frequency of 10% and 5% (the minimum adopted for clinical relevance on tissue specimens). Major issues were reported for low allelic frequency (1%). Interestingly, no false positive results were reported in the wild type specimens.⁴³ In the "second round," slides were created to contain a lower number of cells (2×10^5); the cell lines were engineered to harbour *EGFR* exon 19 p.E746_A750del, *EGFR* exon 20 p.T790M, *KRAS* exon 2 p.G13D, and *BRAF* exon 15 p.V600K.⁴⁴ As in the first round, the major issue was represented by mutations at low allelic frequency (1%). However, in these difficult cases visual inspection of sequencing data was pivotal to avoid missing gene alterations of clinical interest.⁴⁴

In addition, another important issue to take into account that may affect the quality of nucleic acids from cytological specimens is the type of slide adopted. Different slides may be employed. Among these, fully frosted slides enable the highest cell retention and the minimal cell loss during fixation in alcohol-based fixative solutions. In this regard, some studies report that fully frosted slides show better cell retention than positively charged slides, which electrostatically

retain cells on the slide surface, and non-frosted slides. These latter do not feature specialised surface or coating to enhance cellular adhesion and may be useful in high cellularity cases. However, dislodging tumour cells from fully frosted slides, either by scraping or by cell-lifting techniques, is particularly challenging. For this reason, fully frosted slides are less frequently used for nucleic acid extraction.²⁴

Regarding the extraction techniques, scraping and cell lifting are the ones most commonly used. Evidence shows that direct scraping of archival slides achieves a higher nucleic acid yield than cell lifting with Pinpoint solution.²⁴ Interestingly, the use of low-hazard, organic, polymer-based mounting medium EcoMount (BioCare Medical LLC) provides a higher DNA yield than the xylene-based mounting medium Pertex (CellPath).²⁰

6 | CONCLUSION AND FUTURE PERSPECTIVES

NGS is a fascinating technology that has dramatically changed the way molecular predictive medicine is carried out. Since its introduction, it has enabled cytopathology laboratories across the globe to assess vast arrays of different biomarkers in different patients simultaneously, starting from very low nucleic acid input.¹⁰⁻¹² The implication of NGS for targeted treatments in patients with advanced-stage cancers is enormous, when one considers that a high percentage of these patients have only scant cytological samples for morph-molecular purposes.⁹ To circumvent this problem, scientists have resorted to using various cytopreparations, with results that are highly suitable for NGS analysis.^{14,15} Indeed, cytological samples applied to NGS have proven to be a valuable alternative to conventional histological samples both for predicting treatment response and for refining the risk of malignancy. In addition, studies have shown that both CBs and smears are suitable for more complex NGS analysis, such as tumor mutational burden profiling for immune-checkpoint inhibitor administration.^{45,46} Lastly, in an attempt to salvage the limited quantity of cytological specimens, several studies have recently demonstrated the feasibility of adopting supernatant fluids for NGS analysis. Beyond DNA, cytological samples may be also a valuable source of RNA that can be a suitable starting material for NGS analysis. As with DNA, RNA analysis is significantly influenced by several pre-analytical factors. It has been reported that the adoption of non-cross-linking alcoholic reagents may yield superior results in terms of quality and quantity of extracted RNA with respect to formalin.⁴⁷ Interestingly, we have recently reported that our custom SiRe fusion NGS panel is a valid and robust tool for the detection of clinically relevant gene fusions and splicing events in advanced NSCLC patients, starting from RNA extracted from cytological (CB) preparations.⁴⁸ However, in this field of investigation further studies are warranted.

In conclusion, in this review, we briefly highlighted the suitability of cytological samples for NGS analysis in a myriad of cancer types. In particular, NGS analysis may be a valid option for assessing

molecular biomarker status for predictive and diagnostic purposes in patients with advanced-stage cancer. In addition, this review has highlighted that supernatant fluids, typically discarded after cytological sample preparation, represent a valid source of high-quality nucleic acids for NGS analysis.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Pasquale Pisapia, Francesco Pepe, Umberto Malapelle, and Giancarlo Troncone conceived the review. All Authors collected the literature data, wrote the original draft, and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable for this article as no datasets were generated or analysed during the current study.

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REFERENCES

- Salto-Tellez M. More than a decade of molecular diagnostic cytopathology leading diagnostic and therapeutic decision-making. *Arch Pathol Lab Med*. 2018;142:443-445.
- Gambardella V, Tarazona N, Cejalvo JM, et al. Personalized medicine: recent progress in cancer therapy. *Cancers (Basel)*. 2020;12:1009.
- Burke HB. Predicting clinical outcomes using molecular biomarkers. *Biomark Cancer*. 2016;8:89-99.
- Malapelle U, Bellevicine C, De Luca C, et al. EGFR mutations detected on cytology samples by a centralized laboratory reliably predict response to gefitinib in non-small cell lung carcinoma patients. *Cancer Cytopathol*. 2013;121:552-560.
- Lindeman NI, Cagle PT, Beasley MB, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *Arch Pathol Lab Med*. 2013;137:828-860.
- Lindeman NI, Cagle PT, Aisner DL, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the College

- of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch Pathol Lab Med.* 2018;142:321-346.
7. VanderLaan PA. Fine-needle aspiration and core needle biopsy: an update on 2 common minimally invasive tissue sampling modalities. *Cancer Cytopathol.* 2016;124:862-870.
 8. Bellevicine C, Vita GD, Malapelle U, Troncione G. Applications and limitations of oncogene mutation testing in clinical cytopathology. *Semin Diagn Pathol.* 2013;30:284-297.
 9. Bellevicine C, Malapelle U, Vigliar E, Pisapia P, Vita G, Troncione G. How to prepare cytological samples for molecular testing. *J Clin Pathol.* 2017;70:819-826.
 10. Vigliar E, Malapelle U, de Luca C, Bellevicine C, Troncione G. Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective. *Cytopathology.* 2015;26:271-283.
 11. de Biase D, Fassan M, Malapelle U. Next-generation sequencing in tumor diagnosis and treatment. *Diagnostics.* 2020;10:962.
 12. Rothberg JM, Hinz W, Rearick TM, et al. An integrated semiconductor device enabling non-optical genome sequencing. *Nature.* 2011;475:348-352.
 13. Reuter JA, Spacek DV, Snyder MP. High-throughput sequencing technologies. *Mol Cell.* 2015;58:586-597.
 14. Akahane T, Yamaguchi T, Kato Y, et al. Comprehensive validation of liquid-based cytology specimens for next-generation sequencing in cancer genome analysis. *PLoS One.* 2019;14:e0217724.
 15. Velizheva NP, Rechsteiner MP, Wong CE, et al. Cytology smears as excellent starting material for next-generation sequencing-based molecular testing of patients with adenocarcinoma of the lung. *Cancer Cytopathol.* 2017;125:30-40.
 16. Balla A, Hampel KJ, Sharma MK, Cottrell CE, Sidiropoulos N. Comprehensive validation of cytology specimens for next-generation sequencing and clinical practice experience. *J Mol Diagn.* 2018;20:812-821.
 17. Cree IA, Deans Z, Ligtenberg MJ, et al. Guidance for laboratories performing molecular pathology for cancer patients. *J Clin Pathol.* 2014;67:923-931.
 18. Aisner DL, Rumery MD, Merrick DT, et al. Do more with less: tips and techniques for maximizing small biopsy and cytology specimens for molecular and ancillary testing: the University of Colorado experience. *Arch Pathol Lab Med.* 2016;140:1206-1220.
 19. Killian JK, Walker RL, Suuriniemi M, et al. Archival fine-needle aspiration cytopathology (FNAC) samples: untapped resource for clinical molecular profiling. *J Mol Diagn.* 2010;12:739-745.
 20. Dejmek A, Zendeherkh N, Tomaszewska M, Edsjö A. Preparation of DNA from cytological material: effects of fixation, staining, and mounting medium on DNA yield and quality. *Cancer Cytopathol.* 2013;121:344-353.
 21. Roy-Chowdhuri S, Stewart J. Preanalytic variables in cytology: lessons learned from next-generation sequencing-the MD Anderson experience. *Arch Pathol Lab Med.* 2016;140:1191-1199.
 22. Li MM, Datto M, Duncavage EJ, et al. Standards and guidelines for the interpretation and reporting of sequence variants in cancer: a joint consensus recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists. *J Mol Diagn.* 2017;19:4-23.
 23. da Cunha Santos G, Ko HM, Saieg MA, Geddie WR. "The petals and thorns" of ROSE (rapid on-site evaluation). *Cancer Cytopathol.* 2013;121:4-8.
 24. Roy-Chowdhuri S, Chow CW, Kane MK, et al. Optimizing the DNA yield for molecular analysis from cytologic preparations. *Cancer Cytopathol.* 2016;124:254-260.
 25. Pepe F, De Luca C, Smeraglio R, et al. Performance analysis of SiRe next-generation sequencing panel in diagnostic setting: focus on NSCLC routine samples. *J Clin Pathol.* 2019;72:38-45.
 26. Zhang Y, Li J, Hua P, et al. Targeted next-generation sequencing in cytology specimens for molecular profiling of lung adenocarcinoma. *Int J Clin Exp Pathol.* 2018;11:3647-3655.
 27. Karnes HE, Duncavage EJ, Bernadt CT. Targeted next-generation sequencing using fine-needle aspirates from adenocarcinomas of the lung. *Cancer Cytopathol.* 2014;122:104-113.
 28. Reynolds JP, Zhou Y, Jakubowski MA, et al. Next-generation sequencing of liquid-based cytology non-small cell lung cancer samples. *Cancer Cytopathol.* 2017;125:178-187.
 29. Bellevicine C, Sgariglia R, Malapelle U, et al. Young investigator challenge: can the Ion AmpliSeq Cancer Hotspot Panel v2 be used for next-generation sequencing of thyroid FNA samples? *Cancer Cytopathol.* 2016;124:776-784.
 30. Le Mercier M, D'Haene N, De Nève N, et al. Next-generation sequencing improves the diagnosis of thyroid FNA specimens with indeterminate cytology. *Histopathology.* 2015;66:215-224.
 31. Nikiforov YE, Carty SE, Chiosea SI, et al. Impact of the multi-gene ThyroSeq next-generation sequencing assay on cancer diagnosis in thyroid nodules with atypia of undetermined significance/follicular lesion of undetermined significance cytology. *Thyroid.* 2015;25:1217-1223.
 32. Nikiforova MN, Mercurio S, Wald AI, et al. Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. *Cancer.* 2018;124:1682-1690.
 33. Harris T, Sheel A, Zong Y, et al. Cytologically targeted next-generation sequencing: a synergy for diagnosing urothelial carcinoma. *J Am Soc Cytopathol.* 2021;10(1):94-102.
 34. Carrara S, Soldà G, Di Leo M, et al. Side-by-side comparison of next-generation sequencing, cytology, and histology in diagnosing locally advanced pancreatic adenocarcinoma. *Gastrointest Endosc.* 2021;93(3):597-604.e5.
 35. Yamamoto S, Sakamoto Y, Nakano S, et al. Next-generation sequencing assay in salivary gland cytology: a pilot study. *J Oral Pathol Med.* 2020;49:1037-1043.
 36. Fumagalli C, Rappa A, Casadio C, et al. Next-generation sequencing-based BRCA testing on cytological specimens from ovarian cancer ascites reveals high concordance with tumour tissue analysis. *J Clin Pathol.* 2020;73:168-171.
 37. VanderLaan PA, Roy-Chowdhuri S. Current and future trends in non-small cell lung cancer biomarker testing: the American experience. *Cancer Cytopathol.* 2020;128:629-636.
 38. Roy-Chowdhuri S, Mehrotra M, Bolivar AM, et al. Salvaging the supernatant: next generation cytopathology for solid tumor mutation profiling. *Mod Pathol.* 2018;31:1036-1045.
 39. Janaki N, Harbhajanka A, Michael CW, et al. Comparison of cytocentrifugation supernatant fluid and formalin-fixed paraffin-embedded tissue for targeted next-generation sequencing. *Cancer Cytopathol.* 2019;127:297-305.
 40. Hannigan B, Ye W, Mehrotra M, et al. Liquid biopsy assay for lung carcinoma using centrifuged supernatants from fine-needle aspiration specimens. *Ann Oncol.* 2019;30:963-969.
 41. Guibert N, Tsukada H, Hwang DH, et al. Liquid biopsy of fine-needle aspiration supernatant for lung cancer genotyping. *Lung Cancer.* 2018;122:72-75.
 42. Ye W, Hannigan B, Zalles S, et al. Centrifuged supernatants from FNA provide a liquid biopsy option for clinical next-generation sequencing of thyroid nodules. *Cancer Cytopathol.* 2019;127:146-160.
 43. Malapelle U, Mayo-de-Las-Casas C, Molina-Vila MA, et al. Consistency and reproducibility of next-generation sequencing and other multigene mutational assays: a worldwide ring trial study on quantitative cytological molecular reference specimens. *Cancer Cytopathol.* 2017;125:615-626.
 44. Pisapia P, Malapelle U, Roma G, et al. Consistency and reproducibility of next-generation sequencing in cytopathology: a second

- worldwide ring trial study on improved cytological molecular reference specimens. *Cancer Cytopathol.* 2019;127:285-296.
45. Pepe F, Pisapia P, Gristina V, et al. Tumor mutational burden on cytological samples: a pilot study. *Cancer Cytopathol.* 2020. <https://doi.org/10.1002/cncy.22400>. Epub ahead of print.
 46. Alborelli I, Bratic Hench I, Chijioke O, et al. Robust assessment of tumor mutational burden in cytological specimens from lung cancer patients. *Lung Cancer.* 2020;149:84-89.
 47. Bridge JA. Reverse transcription-polymerase chain reaction molecular testing of cytology specimens: pre-analytic and analytic factors. *Cancer Cytopathol.* 2017;125:11-19.
 48. De Luca C, Pepe F, Iaccarino A, et al. RNA-based assay for next-generation sequencing of clinically relevant gene fusions in non-small cell lung cancer. *Cancers (Basel).* 2021;13:139.

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