Next-generation technologies in predictive molecular pathology of lung cancers

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Abstract: A lot of predictive biomarkers have to be tested in advanced non-small cell lung cancer (NSCLC), like *EGFR*, *ALK*, *ROS1*, *BRAF*, *RET*, *NTRK*, and *MET*. According to the 2020 European Society for Medical Oncology (ESMO) guidelines, all patients with advanced adenocarcinoma of the lung should be tested for the above-reported markers, then it is clear that multi-gene approach analyses would be more cost-effective. Aim of this review is to focus on the next-generation techniques currently available for the characterization of lung tumors in clinical practice for predictive purposes. The low amount of material available from fine-needle aspiration biopsy (FNAB) specimens may be successfully overcome by implementing the simultaneous analysis of multiple biomarkers using the same analytical technique. Moreover, liquid biopsy can provide valuable material for the molecular diagnosis of lung cancer. Next-generation sequencing allows for simultaneously screening of multiple markers starting from a small amount of DNA/RNA. Digital polymerase chain reaction (dPCR) is a very highly sensitive method for the analysis of alteration both in biopsies, cytological smears, and in circulating tumor DNA from patients with NSCLC. NanoString nCounter technology is a dual-probe system that allows performing direct profiling of target nucleic acid molecules in a single reaction, without the need for retro-transcription and amplification, and with a very high degree of multiplexing. NanoString has been successfully applied in NSCLC for the detection of rearrangement. In conclusion, nowadays a lot of "next-generation tools" are available for molecular characterization of NSCLC, and the laboratories should apply the best available technique for each specific clinical question.

Keywords: Lung tumors; non-small cell lung cancer (NSCLC); next-generation sequencing (NGS); digital PCR (dPCR); NanoString

Introduction

Nowadays overall incidence of lung cancers in the world is 22.4 cases per 100,000, with a mortality of 18.0 per 100,000, the highest worldwide (*Figure 1*) (1-3).

The principle of targeted therapy consists of the identification of well-defined molecules that can be used as targets for drug treatment, or as molecular markers that play a key role in tumor progression and/or survival. The number of target therapies in non-squamous non-small cell lung cancer (NSCLC) has notably increased since 2013, after that the U.S. Food and Drug Administration (FDA) approved targeted therapy for *EGFR/ALK* mutated lung adenocarcinomas (4).

A number of predictive biomarkers have to be tested in advanced NSCLC, like *EGFR*, *ALK*, *ROS1*, *BRAF*, *RET*, *NTRK*, *HER2*, and *MET* (5,6). In the half of 2021, the first targeted therapy (sotorasib) for patients with NSCLC and *KRAS* p.Gly12Cys (p.G12C) mutation has been approved by the FDA (7). According to the 2020 European Society for Medical Oncology (ESMO) guidelines, all patients with advanced adenocarcinoma of the lung should be tested for the above-reported markers (8,9) (*Table 1*). Even if single-gene biomarker testing is still diffused in clinical practice, it is clear that multi-gene approach analyses would be more cost-effective (10,11).

The development of these advanced techniques led to the concept of "personalized oncology" (12), i.e., each tumor in each person is unique in terms of cause, the form
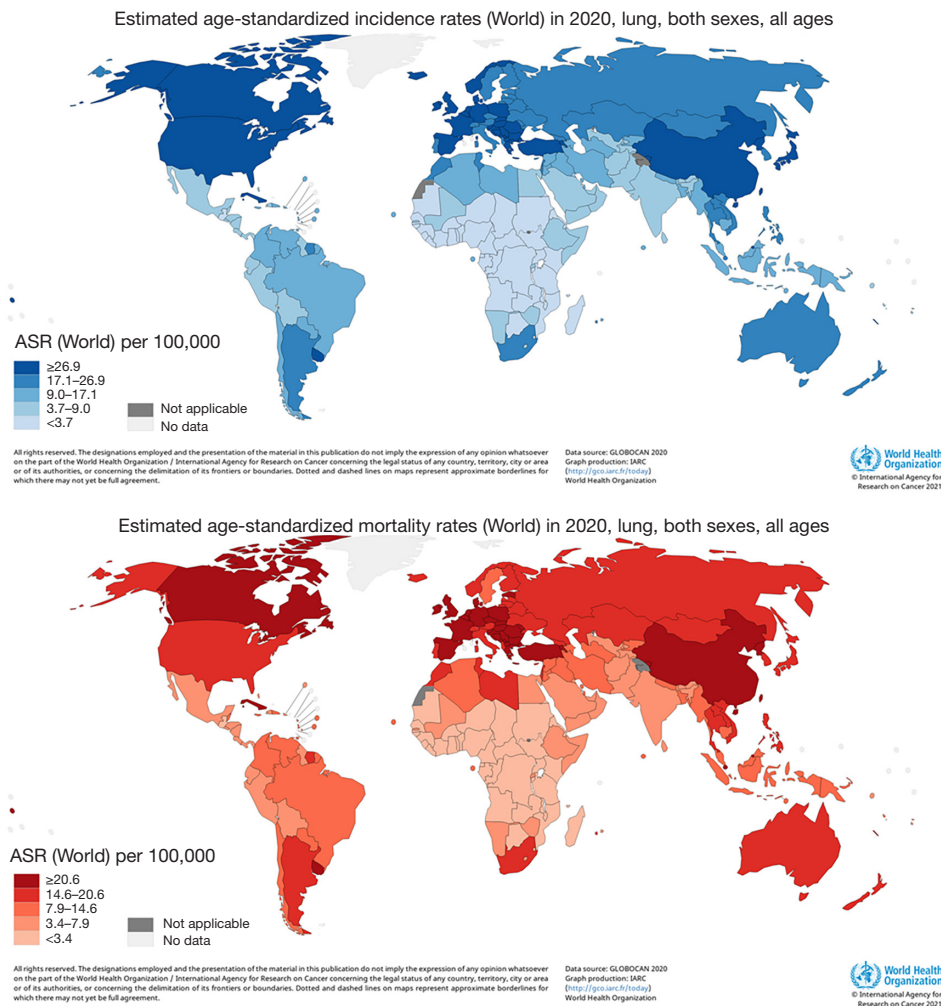

Estimated age-standardized incidence rates (World) in 2020, lung, both sexes, all ages

ASR (World) per 100,000
- ≥26.9
- 17.1–26.9
- 9.0–17.1
- 3.7–9.0
- <3.7
- Not applicable
- No data

Estimated age-standardized mortality rates (World) in 2020, lung, both sexes, all ages

ASR (World) per 100,000
- ≥20.6
- 14.6–20.6
- 7.9–14.6
- 3.4–7.9
- <3.4
- Not applicable
- No data

Figure 1 Incidence and mortality rate of lung cancer worldwide (https://gco.iarc.fr/today) (1-3). ASR, age-standardised rate; GLOBOCAN, Global Cancer Observatory; IARC, International Agency for Research on Cancer.

Table 1 List of genomic alterations and their levels of evidence in NSCLC

Alteration	"Old-fashion" molecular markers	Actual standard of care molecular markers (ESCAT level I)	Future expected markers (ESCAT level II)	Hypothetical future expected markers (ESCAT level III–IV)
EGFR mutations	√	√ (IA, IB)	√ (IIB)[§]	
ALK fusions	√	√ (IA)		
ROS1 fusions	√	√ (IB)		
MET exon14 skipping	√	√ (IB)		
BRAF^{V600E}		√ (IB)		
NTRK fusions		√ (IC)		
RET fusions		√ (IC)		
KRAS	√		√ (IIB)[^]	
MET amplification			√ (IIB)	
ERBB2 amplifications			√ (IIB)	
ERBB2 mutations			√ (IIB)	
PIK3CA				√ (IIIA)
NRG1 fusions				√ (IIIB)
BRCA1/2				√ (IIIA)
FGFR alterations				√
HRD genes				√
MSI				√

[§], *EGFR* exon20 insertion; [^], *KRAS* p.G12C mutation. ESCAT, ESMO Scale for Clinical Actionability of molecular Targets; ESMO, European Society of Medical Oncology; HRD, homologous recombination deficiency; MSI, microsatellite instability; NSCLC, non-small cell lung cancer.

of progression, and response to treatment.

The transition from an old-fashion sequential approach to a multi-gene strategy, due to an increasing number of clinically relevant markers, needs that the technologies used for analysis improved together with the clinical medical needs. The aim of this review is to focus on the next-generation techniques currently available for the characterization of lung tumors in clinical practice for predictive purposes. We considered as "next-generation techniques" those methods that overcome the technical limits of the standard methods usually used in molecular characterization of solid tumors and that have been considered the "gold standard" for several years, such as Sanger sequencing, fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), real-time PCR, pyrosequencing. We then focused our attention on next-generation sequencing (NGS), digital PCR (dPCR)/droplet digital PCR (ddPCR), NanoString, Nanopore, and nonoverlapping integrated read sequencing system (NOIR-SS).

Starting material

Tissue-based specimens

To date, several clinically relevant markers have to be analyzed in advanced-stage NSCLCs (13). This evidence leads to the need of performing several molecular analyses in NSCLC starting from the same specimen, often represented by a low-amount tissue sample. Formalin-fixed and paraffin-embedded (FFPE) specimens are usually the gold-standard material for performing molecular characterization of solid tumors, but this type of starting material is not so commonly available for NSCLCs. In fact, in the majority of routine cases, molecular laboratories dispose of biopsy specimens or cytological material for performing molecular analysis in NSCLC (14-19) (*Table 2*).

The low amount of material available from fine-needle aspiration biopsy (FNAB) specimens may be successfully overcome by implementing the simultaneous analysis of multiple biomarkers using the same analytical technique, as

Table 2 Available material for performing molecular analysis in NSCLC samples

Starting material	Pros	Cons
Surgical FFPE specimens	Huge amount of material	Degradation of nucleic acids due to formalin fixation
	Representative of the lesions	Very rarely available in advanced NSCLC
	Storable in anatomic pathology archives	
Biopsy FFPE specimens	More available than surgical specimens in NSCLC	May be not representative of the lesion
	Storable in anatomic pathology archives	Low amount of material
Cytological smears	Commonly available in NSCLC	Small amount of starting material
	Good quality DNA/RNA	Not archivable
		Usually not accepted for enrolment into clinical trials
Cell block material	Commonly available in NSCLC	Small amount of starting material
	Good quality DNA/RNA	Usually not accepted for enrolment into clinical trials
	Storable in anatomic pathology archives	
Liquid biopsy	Minimally invasive method	Need of very high-sensitive techniques
	Possibility to perform analysis also in patients without biopsy/cytology material	Not feasible for *in situ* techniques
	Serially repeatable during follow-up	Technical variability in the pre-analytical and analytical steps

FFPE, formalin-fixed and paraffin-embedded; NSCLC, non-small cell lung cancer.

NGS. To date, the College of American Pathologists (CAP)/International Association for the Study of Lung Cancer (IASLC)/Association for Molecular Pathology (AMP) guidelines recommend the use of cytological specimens for molecular analysis in advanced-stage NSCLC samples (20).

Liquid biopsy

Liquid biopsy refers to a minimally invasive method of analysis of molecular neoplastic biomarkers [e.g., circulating tumor cells (CTC), circulating tumor DNA (ctDNA), cell-free DNA (cfDNA)] performed starting from any type of patient's body fluid, such as peripheral blood (plasma), bile, urine, saliva, cerebrospinal fluid, and pleural effusion (21,22). In cases in which the lung tumor is inaccessible or the patient's performance status does not allow invasive tissue biopsy, liquid biopsy can provide valuable material for molecular diagnosis (23-26).

The applications of liquid biopsy analysis are several (*Table 2*): (I) diagnosis of lung lesions in patients where it is not possible to obtain pathological material or if the available material is inadequate for molecular analyses; (II) monitoring the treatment response; (III) detection of minimal residual disease. Moreover, the liquid biopsy

procedure can be serially repeated to monitor the development of resistance (27), the development of co-mutations (28), and allows to detect intra- and inter-tumoral heterogeneity that cannot be assessed by analyzing a biopsy specimen at a single site (29). Several scientific studies have demonstrated the efficacy of liquid biopsy in monitoring and characterizing lung tumors (23,30-37), and nowadays liquid biopsy analysis in NSCLC should be routinely integrated into molecular tests currently available in molecular pathology laboratories. If compared to tissue-based specimens, liquid biopsy for NGS has the advantages that can be performed also in those patients where it is not possible to obtain a tissue biopsy, and it can be used for early detection, or disease recurrence using minimally invasive techniques. The analysis performed on liquid biopsy may be an early predictor of response to treatment, allowing to identify also possible acquired drug resistance (32,38). However, it should be taken into consideration that NGS results obtained starting from liquid biopsy specimens require a very careful validation, because of low diagnostic sensitivity in those patients with low tumor burden, the lack of a correlation between molecular results and morphological data (i.e., the type of the lung tumor), and the impossibility to test markers using IHC (e.g., PD-

Table 3 Characteristics of the three main used next-generation techniques in the characterization of NSCLC

Platform	DNA mutation detection	RNA rearrangement detection	Input of nucleic acid	Sensitivity	Multiplexing (number of markers)
NGS/massive parallel sequencing	+++	++ (amplicon based)/+++ (capture based)	10–20 ng (amplicon based)/ 200 ng (capture based)	0.1–5%	++/+++ (depending on panel)
dPCR	+++	++	2–50 ng	0.1–1%	+
NanoString	N/A	+++	100–200 ng	>1.5 fold (>5 copies per cell)[†]	+++ (but only for gene fusion)

[†], at least 50% of tumor cells. +, suitable for. NSCLC, non-small cell lung cancer; NGS, next-generation sequencing; dPCR, digital polymerase chain reaction; N/A, not available.

L1 expression) (32,38).

Analytical techniques

NGS

NGS, also called massive parallel sequencing (MSP), is a multigene mutational assay that can simultaneously screen multiple markers starting from a small amount of DNA/RNA. The low amount of nucleic acid input makes NGS a suitable technique for molecular analysis in lung biopsies and cytological samples. Adequacy of samples for NGS analysis is assessed according to tumor cellularity and enrichment in neoplastic cells. Both factors may, however, vary depending on the analytical sensitivity of the platform used (39). NGS allows starting not only from FFPE specimens (both biopsy and surgical specimens), but also from cell block preparations, cytological smears, liquid-based cytology (LBC), and fluids of fine needle aspirate procedure (19,40-43). The minimum amount of nucleic acid varies according to the platform and panel used, but usually ranges from 10 to 50 ng for DNA or RNA input for amplicon-based methods, and up to 200 ng for capture-based panels. As a general rule, before clinical applications, laboratories performing NGS must validate the adequacy of the entire pipeline analysis, from acid nucleic extraction to the interpretation of the output results (41,44,45).

ESMO guidelines highlight the key role of NGS in the molecular characterization of patients with NSCLC in clinical practice (5), stating that "*If available, multiplex platforms (NGS) for molecular testing are preferable*" (5). In fact, NGS allows the simultaneous detection of *EGFR* and *BRAF*, *RET*, *NTRK*, *ALK*, *HER2*, and *ROS1* rearrangements, and *MET* exon skipping, other than other potentially useful biomarkers (as *KRAS* mutations). NGS is not only a multi-gene multi-patients technique but allows to perform these

multiple tests at a very high depth of coverage (*Table 3*). This high analytical sensitivity is crucial mainly in those samples with a low enrichment in neoplastic cells. Using NGS panels it is then possible to investigate a set of genes in a single test, able to identify alterations even in the scarce biopsy tissue often available in everyday practice (46).

NGS technology allows the analysis of DNA alterations, copy number aberrations (CNA), and gene translocations in the same run. NGS panels usually used for lung-adenocarcinomas should then allow detecting at least the markers that are nowadays available for targeted therapy, such as *EGFR/KRAS/BRAF/MET* exon14 skipping mutations, *ALK/ROS1/RET/NTRK* rearrangements in lung adenocarcinoma (5).

Profiling all markers in the same NGS "run" allows for defining the more appropriate treatment for the NSCLC patients. In fact, using a single analysis it is possible to determine if a patient should be treated using EGFR tyrosine kinase inhibitors (EGFR-TKIs) (gefitinib, erlotinib, afatinib, osimertinib, and dacomitinib) in tumors with *EGFR* mutations, using ALK-/ROS- or RET-inhibitors (in patients with NSCLC harboring *ALK/ROS1/RET* rearrangements), BRAF inhibitors (dabrafenib, vemurafenib) if *BRAF* activating mutations are detected, or using NTRK inhibitors (in patients whit tumors harboring *NTRK1-2-3* rearrangements).

The multi-gene NGS approach avoids performing several tests for different markers. In fact, an alternative to the NGS approach would force to perform real-time PCR for *EGFR* and *BRAF* point mutations and real-time PCR and/or *in situ* techniques [i.e., IHC—and/or in situ hybridization (ISH) techniques] for *ALK/ROS1/NTRK/RET* rearrangements.

Several multi-gene panels have become commercially available in recent years. These panels may be designed for
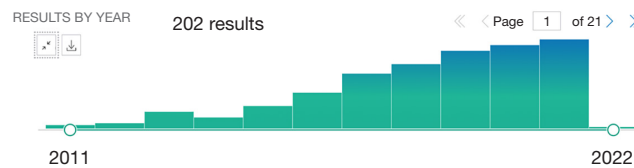
Figure 2 Number of scientific articles published on Next-generation sequencing in lung tumors (source: PubMed; Query: "(Next generation sequencing[Title]) AND (Lung[Title]) AND (cancer[Title] OR carcinoma[Title])").

investigating specific markers for specific tumors or include a very high number of targets ("comprehensive" panels). Moreover, the NGS approach allows the set-up of custom/laboratory-developed multi-gene panels for the selection of targets, according to the needs of the medical community, serviced by the molecular laboratory (43).

Hybridization capture and amplicon sequencing are the two most common types of NGS panels used in clinical practice. In the hybridization capture panels, target sequences are captured using Biotinylated probes, while in the amplicon-based panels a first PCR amplifies the desired target sequences with specific primers (47). The great advantages of hybridization capture panels are the scalability and the detecting of gene rearrangements without requiring prior knowledge of fusion partners. On the other hand, hybridization capture protocols are usually more laborious and may be inadequate in case of a low quantity of acid nucleic availability. Amplicon-based panels are characterized by a simpler and faster workflow and allow to obtain evaluable results also starting from a low amount of nucleic acid (up to 10 ng per reaction). The amplicon-based panels are very efficient in DNA mutation detection but may suffer from PCR bias and are not optimal for the identification of gene rearrangements because these panels allow detecting only previously known rearrangements that have been included in the primers pool.

The potentially very high analytical sensitivity of MPS allows the investigation of the actionable mutations in NSCLC patients also starting from liquid biopsy/cfDNA (48-51).

Another interesting point about the NGS approach for lung tumor analysis is that multi-gene panels allow analyzing also markers that nowadays are not in clinical practice but that would allow driving patients onto therapeutic trials (5). The analysis of hundreds of targets simultaneously has been defined as comprehensive genomic profiling (CGP) (4,52). If on one hand using CGP multiple actionable targets can be identified with small amounts of samples (thereby improving the success of the tests), on the other hand, the clinician had to manage a lot of possible

alterations also for genes considered as "non-actionable targets". However, it has been hypothesized that the routine use of CGP for NSCLC would: (I) improve the likelihood of obtaining evaluable samples for analyses, decreasing the need to obtain new specimens; (II) CGP tests are probably more sensitive for identifying actionable targets in routine tumor samples than traditional sequential testing techniques (4).

Since 2011 more than 200 scientific papers have been published about the use of NGS in lung tumors, with an ever-increasing number in the last years (*Figure 2*). Turnaround time (TAT) is a crucial aspect in the management of molecular tests of NSLC. Guidelines by CAP/IASLC/AMP, as well as local guidelines (20), recommend that molecular testing TAT should not exceed 10 working days. However, in real-world clinical practice, some delays in NGS TAT may be observed. Molecular laboratories must be equipped with different platforms, that prove themselves to be useful in case of NGS would fail due to pre-analytical tissues or for orthogonal confirmation. In fact, single-gene testing approaches may be adopted as orthogonal techniques useful to confirm challenging cases, while broader NGS testing for patients with advanced NSCLC should be the best strategy (53,54). The cost of a multi-gene panel for NSCLC is strongly dependent on the choice of the panel. However, a recent study about the feasibility of NGS in NSCLC revealed a median cost for reagents of about €500 per sample (55). Moreover, comparing the total cost per patient (i.e., reagents, consumables, personnel time, equipment investment and maintenance, and overheads) the authors have observed that the median cost for the NGS strategy was lower (about €1,400) if compared to that of standard strategies (about €3,000) in the current NSCLC scenario (55). These differences are going to increase if we consider the increasing number of useful markers in NSCLC. To date, ESMO guidelines recommend that an NSCLC specimen is profiled using NGS. Moreover, the guidelines suggest that those medical centers that perform development programs and clinical trials use multigene sequencing in the

context of molecular screening programs, even if it should be considered that using NGS it is not possible to evaluate the protein expression and localization (e.g., it is not possible to evaluate PD-L1 expression) (5). In the future, the role of NGS multi-gene testing will be always more crucial for the characterization and clinical management of advanced NSCLC. In fact, the number of emerging biomarkers that need to be tested in the clinical practice of NSLC is expected to highly increase within the next 3 years, including for example microsatellite instability (MSI), *FGFR*, *BRCA*, and homologous recombination deficiency (HRD) genes (*Table 1*).

dPCR/ddPCR

dPCR is a very highly sensitive method for the analysis of alteration both in solid tissue specimens (biopsy or cytological smears) and in ctDNA from patients with NSCLC (56,57). The dPCR maybe then also used in liquid biopsy specimens for therapy monitoring of NSCLC patients (57). dPCR has been successfully used for ctDNA detection of *EGFR* mutations in advanced lung adenocarcinomas (48,49), allowing to detect mutations in cfDNA with a fractional abundance of at least 0.1% (58-82) (*Table 3*). dPCR may be also used for quantitative measurements of T790M mutant copy number in plasma cfDNA to predict treatment response and survival outcomes in NCSLC patients (48,83,84).

dPCR also allows the use of multiplex assays for simultaneous detection of multiple *EGFR* tyrosine-kinase inhibitor-sensitizing mutations (85-90). Interestingly, using dPCR it has been demonstrated that the detection rates of *EGFR* mutations were higher in bronchial washing fluid than in the plasma of patients with lung adenocarcinoma (91). Even if nowadays ddPCR has been mainly used for *EGFR* detection in cfDNA, this technique may also be used for the detection of alterations in other clinical biomarkers, such as *ALK* alterations, *KRAS* mutations, and *Myc* amplification (88,92-96). Intriguingly has been used for quantifying PD-L1 levels in NSCLC biopsy specimens (97). As reported above, liquid biopsy refers to any type of patient's body fluid. dPCR has been used for evaluating *EGFR* mutations in sputum samples of patients with advanced *EGFR*-mutated NSCLC (98).

NanoString

NanoString nCounter technology is a dual-probe system that allows performing direct profiling of target nucleic acid molecules in a single reaction, without the need for retro-transcription and amplification, and with a very high degree of multiplexing (99-101). Using NanoString nCounter it is possible to analyze up to 800 target genes per reaction. The quantification of target molecules is performed without reverse transcription or amplification steps. In this way, it is possible to obtain faithful and reliable data not influenced by any type of bias, inevitably introduced by enzymatic reactions. This analysis can be then performed starting from degraded clinical samples, as tissues FFPE specimens, allowing the numerous samples preserved in the archives of pathological anatomies to be used retrospectively. In fact, the direct counting of messenger RNA (mRNA) molecules leads NanoString to be less sensitive to the preanalytic treatment of samples (102). The advantages of this technology are (*Table 3*): (I) the low amount of input RNA (less than 200 ng) needed for the evaluation of expression of a huge number of genes; (II) the evaluation of gene rearrangement does not require prior knowledge of fusion alterations.

The NanoString technology represents then a potentially useful genomic platform for the detection of gene fusions in clinical practice with high sensitivity, reproducibility, and technical robustness (99).

In NSCLC NanoString technology has been successfully used to characterize druggable rearrangements as those in *ALK*, *ROS1*, and *RET* genes, with high accuracy and sensitivity (103-108).

NanoString analysis performed on a cohort of 214 lung squamous cell carcinomas detected no *ALK*, *ROS1*, or *RET* gene rearrangements, confirming that these rearrangements are very rare in lung squamous cancer (109).

NanoString has been successfully applied in NSCLCs for the detection of rearrangement not only in FFPE but also in cytological specimens (102). The efficacy of NanoString also on cytological specimens put this platform even more as one of the molecular tools applicable in the routine practice of lung cancer.

Other techniques

Nanopore

Nanopore is one of the so-called third-generation sequencing techniques allowing a single molecule of nucleic acid (DNA or RNA) to be sequenced without the need for PCR pre-amplification. Nanopore works recording a sequence-dependent electrical signal when DNA molecules pass through a pore. To date, Nanopore technology is used in clinical practice for rapid identification of viral pathogens,

environmental and food safety monitoring, plant genome sequencing, and monitoring of antibiotic resistance (110). Nanopore technology is nowadays optimized for long-read sequencing, and then not ideal for analysis from *ex vivo* neoplastic samples, but in the next future could be also implemented in the characterization of tumors in the pathology laboratories. In a recent study performed on cfDNA from 6 lung cancer patients and 5 healthy subjects, Martignano and colleagues demonstrated the technical feasibility of Nanopore sequencing for copy number variation (CNV) analysis of short plasmatic cfDNA (111).

NOIR-SS

NOIR-SS is a sequencing method that uses molecular barcodes. This method allows a high-fidelity target sequencing system of individual molecules in plasma cfDNA (112). NOIR-SS enables a more accurate quantification and measurement of allele fractions than conventional barcode sequencing by removing erroneous barcode tags during data analysis and then reducing the number of false positives. NOIR-SS technology has been successfully adopted for targeting *EGFR* p.L858R ctDNA mutation in plasma of patients with lung adenocarcinoma (113).

Conclusions

The advent of next-generation techniques (e.g., NGS) into clinical practice of lung tumors has allowed to introduce molecularly driven treatment choices. Moreover, an integrated morphological and molecular characterization of the lung tumors is now performed for the diagnosis. It should be then considered that it has become increasingly crucial to have sufficient material for histological, immunohistochemical, and molecular characterization. For this reason, it is mandatory to dispose of highly sensitive and specific techniques that allow the molecular characterization of the lung tumors in a robust way starting also from a low amount of tissue specimens. In our experience, to date, the multi-gene approach using an NGS technique is preferable to sequential testing. The proper application of the best suitable technique for specific clinical requests and for the availability of biological material provides a powerful tool to laboratory and physicians for having the best management of patients affected by lung tumors.

Acknowledgments

Footnote

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