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Predictive markers in lung cancer: a few hints for the practicing pathologist

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Summary

This review article highlights some important points in the evolving area of predictive biomarkers determination in non-small-cell lung cancer toward standardization of testing practices, including *EGFR* mutations, *ALK* and *ROS1* rearrangements and immunohistochemical expression of PD-L1. Considerations for selecting appropriate populations for molecular testing, and emergence of other targetable molecular alterations are also discussed.

Introduction

The rapid development of molecular biology in recent years has allowed us to understand many molecular steps involved in the development and progression of lung cancer [1-3]. The identification of molecular alterations in specific tumor genes that function as key drivers for neoplastic growth has laid the foundations for new therapeutic approaches with targeted agents. An accurate detection of target alterations is therefore mandatory for an efficient treatment [4].

These new developments have changed the way pathologists now must deal with routine lung cancer diagnostic: the task is not only to identify and classify lung neoplasms, in most cases using very small bioptic or cytological samples, but include also the evaluation of several molecular targets, which have to be investigated with several different technologies [2 5 6]. Some of these targets have to be evaluated using a variety of sequencing techniques (such as *EGFR* mutations), while other have to be investigated with in-situ immunohistochemical and/or hybridization methods (such as *ALK*, and more recently *ROS1* translocation) [2 7]. As a further complexity factor, the recent introduction of immune check point modulators in lung cancer therapy, requires the evaluation of other biomoloecular markers, such as PD-L1 [8].

In this brief paper, we will synthetically focus on some of the aspects of the process of evaluating these molecular targets, trying to provide a rapid answer to some of the most frequent questions which may arise in routine practice. For more detailed analysis of the various markers one should refer to the many exhaustive reviews and position papers available in literature.

Tissue is the issue

How should we manage small endoscopic biopsy samples for molecular analyses?

To maximize the diagnostic yield, endoscopist should be encouraged to perform multiple sampling of the lesions (ideally 5 samples) and these tissue fragments must be managed in an extremely conservative way. The tissue

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fragments obtained for a given patient can be subdivided into two paraffin blocks and should be minimally sectioned in order to maximize tissue availability for further additional studies. If immunohistochemical analyses are needed for diagnostic purposes, they must be kept at the minimum: TTF1 and p63/p40 are the only needed makers to sub-classify non-small cell lung cancers (NSCLC) which cannot be better classified based on morphology alone [9].

A poorly differentiated NSCLC lacking TTF-1 and p63/p40 expression should be defined as NSCLC n.o.s. (non-squamous NSCLC is acceptable) and follow the molecular and therapeutic steps of adenocarcinomas (9). Once the diagnosis is done and a molecular profile is needed, the pathologist must evaluate if the tissue samples are adequate for molecular tests and select the most representative material to be used for DNA sequencing techniques and for immunohistochemistry and FISH analyses. If concurrent cytological samples, especially cell-blocks (see next paragraph), are available, the pathologist should select which of the samples is more cellular and appropriate for biomarker analysis [10 11].

Alternatively, it is possible to perform upfront multiple sectioning of the paraffin blocks, to obtain multiple sections for diagnostic and predictive IHC staining and FISH analyses, and sequencing techniques [11]. This approach avoids the need of re-cutting the blocks with the danger of misalignments on the microtome during the re-cutting procedure, which could lead to loss of precious tissue. Recently, special microtomes have been produced which allow a precise orientation of the actual cutting surface of the tissue block, allowing to overcome the above problem of the microtome misalignments problems [11].

Whenever serial sections are obtained for sequencing techniques, it is important to collect them on glass slides to control that the content of tumour cells is homogeneous in all sections; frequently it is enough to stain the last section and to check its tumour content; alternatively, if the material is very scarce or tumour cells are few and admixed with abundant normal tissue, all sections need be stained and checked. If the tissue section contains and admixture of normal and tumoral tissue it is possible to microdissect the tissue sections to focus the analysis on a more representative sample enriched in tumour cells.

Several other tricks can be adopted by pathologists in routine practice to optimize tumor tissue handling: for bony metastasis it is useful to embed specimen fragments devoid of bone in separate blocks to avoid decalcification of tumour cells, which is important to preserve DNA integrity [10 11]; stained or immunostained slides can be re-used; rapid-on site evaluation (ROSE) of fine-needle aspiration procedures during bronchoscopy or trans-thoracic biopsy may increase the yield of tumor cells [12-16].

Are lung cancer cytological samples appropriate for biomarker testing?

All kind of cytological samples, including previously stained air-dried or alcohol-fixed smears and liquid-based (LBC) samples and cell blocks (CB), can be used for molecular analyses, providing that the quantity and percentage of tumour cell is adequate. Rapid on-site evaluation (ROSE) usually improves the quality of the procurement procedure, allowing to obtain more adequate cellular samples, with higher tumour fraction [11-16]. Alcohol fixed cytological samples may even offer a better-quality DNA than formalin fixed bioptic material. CB are a very valuable source of tumour material, but are subject to some variability in their preparation techniques across institutions and their use is very much dependent upon these technical aspects [2 5 10 11 16-20]. The main advantage of using CB is that they can be managed similarly to bioptic samples, and it is relatively easy to acquire multiple serial sections to perform both in situ and sequencing assays. Recently, an automated device to prepare CB from liquid based cytological samples has been introduced in the market and could probably overcome some of the issues of interlaboratory efficacy in preparing CB [21 22].

When smears are the only material available for DNA extraction for molecular studies, the slides should be digitally scanned or photographed to record the cytomorphology of representative diagnostic microscopic fields for the archives. A written permission by the patients should be obtained for these procedures.

Smeared cytological specimens are adequate for in situ analysis of *ALK* and *ROS1* translocation using FISH assay but not all authors agree that they are also suitable for ALK or ROS1 IHC staining. CB may allow both FISH and ICH analyses of *ALK* and *ROS1* translocation [2 5-7 10 11 17 23].

Immunohistochemical predictive pathology

Which predictive immunohistochemical test should be used and in which order should we order them?

In routine practice, only ALK and ROS1 (in non-squamous NSCLC) and PD-L1 (in all NSCLC) must be immunohistochemically evaluated, either as a screening method to be followed by additional confirmatory investigations using FISH or as a definitive tool to define the status of the marker. ALK and ROS1 evaluation are required to select patient to be treated with crizotinib while PD-L1 is required for selecting patients to be treated with pembrolizumab [24-28]. There is a debate concerning whether ALK and ROS1 immunostains in non-squamous NSCLC should be performed after *EGFR* mutation analysis or if they should be done independentely from *EGFR* analisys. According to some authors [7], ALK and ROS1 should be evaluated only in *EGFR* wild type cases, or even more conservatively only in *EGFR* and *K-RAS* wild type cases. However anecdotal cases of co-existence of *EGFR* mutation and *ALK* translocation

can suggest that *ALK* evaluation and *EGFR* sequencing should be done concurrently [2 10 11 29-31]. PD-L1 immunohistochemical evaluation should be done to identify patients to be treated with immune-check point modulator pembrolizumab as first line therapy for *EGFR*, *ALK* and *ROS1* negative metastatic non-squamous carcinomas and for all metastatic squamous cell carcinomas; the cut-off for defining a case as positive is 50% of tumour cells. Pembrolizumab can be used also in second line setting, after failure of other therapies, but the PD-L1 cut-off in this case is much lower (> 1% of tumor cells) [28 32 33].
Whether ALK, ROS1 and PD-L1 immunostaining should be performed as a combined panel or in a given sequence, and if they should be done upfront on all newly diagnosed cases or as the result of a specific request of the oncologist, should be defined by each laboratory on the basis of the local organization.

Which are the best reagents for ALK immunostaining?

ALK immunostaining can be done using at least two different monoclonal antibodies, 5A4 and D5F3. Both antibodies give optimal results, and can be used on a dedicated platform (such as with the D5F3 Mab used on the Ventana platform, which has been FDA approved as a companion diagnostic for crizotinib) or as a laboratory determined tests (LDT), provided that internal and external controls are properly managed [2 27 28 32-39]. The easiest available control for ALK immunostaining is the normal human appendix, whose ganglia show a positive granular cytoplasmic ALK immunostaining. The evaluation of the immunostaining is based on a two-tiered system for the D5F3 antibody used on the Ventana platform as a FDA approved kit, or on a 4-tiered system, 0 to 3 plus, for the 5A4 (used on any platform) and D5F3 used with platforms other than Ventana's; using this approach, cases scored 2 plus must be submitted to FISH confirmatory test [35 39]. A FISH confirmatory test is also suggested for 3 plus scored cases with 5A4 and D5F3 used with platforms other than Ventana's. Recently, another monoclonal primary antibody, namely clone 1A4/1H7 (OriGene Tech.) showed great concordance results when compared with FISH testing and the other aforementioned clones [36].

Which are the best reagents for ROS1 immunostaining?

Up to now there is only one monoclonal antibody which has been sufficiently characterized and widely used, which is the D4D6 [2 23 24 26 27 35 40-45]. A new antibody (EPMGHR2) has recently been introduced to the marker, but literature and EQA data are still lacking. The D4D6 antibody can be used as a LDT to screen cases to be submitted to confirmative FISH analysis, provided that internal and external controls are properly managed. There is no universally accepted scoring system for ROS1 expression, and literature reports describe two methods, either a 4-tired scoring system (from 0 to 3 plus, and any degree of staining is regarded as positive) or a histo-score system (H) based on the percentage and intensity of staining of tumour cells (cases with a H > 100 are considered positive) [41 45]. All positive cases must be confirmed with FISH analysis. Although ROS1 can be occasionally expressed by normal pneumocytes and macrophages, it is usually not expressed in normal human tissues: therefore, a search of a positive lung tumour to be used as a positive control is pivotal [23]. The most rewarding way to search for these tumors, beside exchanging material between institutions, is to perform multiple immunohistochemical and FISH analyses for ROS1 on tissue microarrays (TMA) of lung adenocarcinomas, enriching the TMA cancer population with tumors from young, non-smoker women with advanced diseases, which are the category most frequently positive for *ROS1* rearrangements [46].

Which are the best reagents for PD-L1 immunostaining?

PD-L1 immunostaining is pivotal in the selection of patients to be treated with specific PD1/PD-L1 inhibitors [27 28 32 33 49]. Several anti-PD-L1 antibodies and different scoring systems have been proposed for PD-L1 testing on NSCLC biomaterials. In the clinical trials using nivolumab, the analysis of immunohistochemical expression of PD-L1 through the 28-8 monoclonal antibody (Agilent, Santa Clara, CA, USA) was assessed using different thresholds of tumoral cell (TC) positivity (1%, 5%, and 10%), with significant results in terms of responsiveness to the treatment for cases with TC positivity ≥ 1%. In the case of pembrolizumab, the trials, using the different anti–PD-L1 Agilent clone 22C3, support the use of a threshold of 1% (for second line treatment) and 50% (for first line treatment) of PD-L1 expressing TCs. The clinical trials on durvalumab, using the SP263 clone on the Ventana platform, suggest a cut-off of 25% of PD-L1 positive TC. Finally, the companion test for atezolizumab, using the SP142 clone on the Ventana platform, requires the assessment of both TC and/or tumor-associated immune cells (ICs); in particular, for TCs, 4 different grades of staining should be considered (TC0-3), defined around cut points of 1%, 5%, and 50% TC staining; for ICs, the area of tumor infiltrated by PD-L1– expressing ICs should considered as a percentage with cutoffs at 1%, 5%, and 10% (IC0-3). Currently, the correlation between the relative specificity and sensitivity of the different clones has been widely explored in literature, and several studies have shown that the clones SP263 on Ventana platform and 22C3 and 28.8 clones on Agilent platform may offer similar results when investigating PD-L1 expression in tumor cells. From a practical point of view, according to Italian regulation for pembrolizumab therapy, pathologists must identify PD-L1 positive cases using a robust and reliable test, but no cogent indication is given as to the test to be used. Actually, the only FDA approved systems for selecting patients to be treated with pembrolizumab are the 22C3 clone on Agilent platform (PharmDX, approved on the basis of clinical efficacy) and

the clone SP263 on Ventana platform (approved on the basis of laboratory equivalence with the 22C3 Agilent system). The same undiluted monoclonals can be used on different platforms, as laboratory determined tests (LDT), but accurate validation should be performed by each laboratory to assure that the LDT test has the same performance of the approved systems. The problem of accurate validation of LCT is underscored by the comparison between results of the first NordiQC run C1 in 2017 and the last C3 run in 2018. In the first run LDT assays showed a pass rate of 20%, while in the last run LDT assays, showed a pass rate of 91%. These results, although indicating the challenge to validate a reliable LDT protocol to give concordant results, are promising and open the possibility to develop reliable LDT tests.

Which sample should be used for PD-L1 testing?

In clinical trials, PD-L1 immunohistochemical evaluation has been mainly performed on surgical samples. Since heterogeneity of PD-L1 expression is a well known phenomenon, its evaluation on small biopsy and cytological samples still needs to be fully validated. However preliminary, and limited, data show that bioptic samples and CB may me representative of PD-L1 status of the tumour and can be reliably used in routine diagnostic. Recent data also support the use of CB obtained from alcohol fixed cytological samples [50]. In this perspective, the scoring system based on the SP142 clone cannot be used on cell blocks and cytospin, because of the need of ICs evaluation. Finally, the variable and dynamic PD-L1 expression in tumor cells, also related to its inducibility by several factors, including previous chemotherapy and pre-analytic issues, should point on re-biopsy of recurrent disease in order to provide a more reliable profile of the biomarker.

PD-L1 epitopes are also potentially unstable with prolonged specimen fixation or inadequate tissue handling before fixation, or prolonged block storage [51].

FISH for predictive pathology

Which FISH test should be used?

In routine practice, only those markers which are defined as companion diagnostics of a given approved drug must be evaluated. This means that up to now, in Italy, only *ALK* and *ROS1* rearrangements must be evaluated with FISH either as an upfront test to define the status of the marker or as a confirmatory test of a previous positive immunohistochemical screening method.

The break-apart FISH test for *ALK* is indicated as a reflex test for the determination of *ALK* fusions in cases with doubtful results obtained at IHC. In addition, the FISH test for *ALK* is indicated for those cases where smeared cytology is the only available sample, as ALK IHC on smeared cytology may not always be reliable. FISH remains still the gold standard for the exact determination of the number of rearranged cells and the type of rearrangement (translocation *vs* 5' deletion) even though this information are not clinically relevant thus far. Beside FISH, there are also commercially available kits for chromogenic ISH for *ALK*. The problem of chromogenic ISH in fusions is the challenge in calling the break-apart signals since the chromogens used in the kits have variable reactivity to the revelation systems. These variations result in different sizes of the chromogenic dots and therefore make difficult the call of split signals. At this time, chromogenic ISH is not recommended for the detection of *ALK* fusions.

The break-apart FISH test for *ROS1* is the gold standard method for the assessment of ROS1 fusions in NSCLC. FISH can be used upfront or as a confirmatory test of a positive immunohistochemical ROS1 result.

FISH analysis can be performed with commercially available break-apart, dual-color, FISH IVD tests and a number of other kits for research use only (RUO). IVD tests are preferable, although currently available kits are not coupled with automated ISH instruments, and must be used manually or semi-automatically, leaving certain variability as part of the procedures. RUO reagents could be validated in molecular pathology laboratories as LDT, through internal and external quality controls. LDT FISH tests based on homemade probes are generally discouraged for diagnostics. IVD approved kits are generally ready-to-use. They come with optimized pre-mixed probes, positive and negative control slides and reagents for slide preparations. Critical steps for the success of FISH are the pre-analytical conditions (tissue characteristics, fixation and processing), sample preparation (section oven-baking, deparaffination, and protease digestion). Protease digestion is critical since it may vary upon tissue characteristics and it is not easily standardized even using the dedicated reagents suggested by the kits. Reading of FISH is perhaps the most challenging variable. IVD tests provide cut-offs for break-apart signal counting and reporting. Unfortunately, FISH is generally read by eye with few exceptions in selected centers. This makes FISH reading and reporting very subjective. Two operators must read any diagnostic FISH blindly. One of the two readers must be a pathologist for the histological recognition of actual tumor cells. High expertise of the two readers in diagnostic FISH is therefore highly required for final reporting.

It is important to note that some break-apart FISH assay would fail to detect rare gene fusions, as *GOPC-ROS1*, because the 5' probe overlaps or includes *GOPC*, then leading to false negative results [23 58].

Should FISH be performed as frontline test?

Frontline FISH has been generally discontinued due to the implementation of approved immunohistochemical reagents, which outperform FISH in terms of throughput, time of accomplishment and interpretation of the results. FISH is generally recommended as a confirmatory test of borderline results or positive results obtained with RUO immunohistochemical reagents [23].

FISH is time-consuming, requires dedicated instruments (mostly the fluorescence microscope and a dedicated image analysis software), and expertise of operators and should be performed in reference laboratories to cut costs of dedicated reagents, instruments and human resources.

Technological platforms

Should we abandon IHC and FISH for more advanced molecular platforms?

In current clinical practice, tumor molecular profiling involves multiple assessments (immunohistochemistry, fluorescence in situ hybridization and sequencing), most of which target a single gene or type of mutation or a limited panel of gene alterations, resulting in increased costs and turn-around time. It is reasonable to foresee that these techniques will be soon replaced by methods based on massive parallel sequencing (so called next generation sequencing, NGS technology)[59-63]. The higher throughput of NGS allows analyzing a very large number of gene somatic mutations as well as fusions and copy number variations starting from low amounts of extracted DNA and RNA. There are commercially available NGS-based panels able to provide information about all the most frequent somatic and germline mutations and fusions in solid tumors at the same time in multiple patients, at reasonable costs. Different NGS-based approaches (targeted NGS, whole exome and whole genome NGS) exist and are used for different applications. In diagnostic pathology laboratories, targeted NGS (amplicon-based) is widely applied. The reason of this choice is that the vast majority of sequence information generated by whole exome/genome sequencing cannot be translated into therapeutic options and that whole genome sequencing needs unfixed samples and a complex bioinformatics approach considering both germinal and somatic data.

Targeted-NGS is a complex technique that must be controlled in every step and supervised by well-trained technicians, molecular biologists and pathologists to avoid the production of unreliable data and a misinterpretation of the results. The use of approved assays offers advantages: they comprise clinically actionable genes, are manufactured according the GMP standards and strict quality controls, and do not need a validation process, as requested by RUO assays but simply a performance verification. Quality assurance and internal and external quality control procedures should be strictly followed in order to offer the patients the best therapeutic options.

How important are different sensitivities of the different platforms?

Sensitivity of different technological platforms is reported in the following Table I. However, all the platforms performances must be considered in the context of the laboratory activity: number and types of targets requested by the oncologists, turn-around time, personnel skilling and economic sustainability.

In general, a sensitivity of 1 to 5% is considered acceptable and the procedure should be conducted according to the laboratory standard operating procedures. Although tissue specimens are considered preferable for molecular testing, adequate cytologic samples can also be successfully used.

What should be done with data obtained using multiplex systems which can provide informations about non-druggable genes?

New techniques allow the detection of several gene alterations, but apart from a restrict series of well-known genetic aberrations that clearly impact on prognosis or candidate patients to validated targeted therapies, the identification of actionable variants remains a challenge.

In fact, there is limited evidence of the clinical significance of most genetic variants and there is a lack of a comprehensive database of genetic variant-phenotype associations. The existing catalogs of clinically actionable variants are not standardized, are maintained by different entities (e.g., laboratories or government organizations), and are not designed to interact with Elec-

Tab. I. Techniques for detecting gene mutations: applications and sensitivity.

Method Sensitivity	(% mutant DNA)	Mutations	Multiplex	Applications
Sanger	10-25%	Known/new	No	Tissue
Pyro	5-10%	Known	No	Tissue
Multiplex	5%	Known	Yes (HS)	Tissue
PCR Snapshot				
Cobas	3-5%	Known	No	Tissue/plasma
MALDI-TOF	1-10%	Known	Yes (HS)	Tissue/plasma
NGS	1%	Known/new	Yes	Tissue/plasma
Therascreen	1-5%	Known	No	Tissue/plasma
Scorpions ARMS	1%	Known	No	Tissue/plasma
LNA clamp	1%	Known	No	Tissue/plasma
BEAMing	< 0.1%	Known	No	Tissue/plasma
Digital droplet/PCR	< 0.1%	Known	No	Tissue/plasma
CAPP Seq	< 0.02%	Known/new	Yes	Plasma

tronic Health Records. Moreover, the increased scale of data generation has made analysis and interpretation of NGS data complex and often rate-limiting in the overall workflow. To address this bottleneck there are web-based applications for research use that streamlines the identification of relevant published evidence associated with gene variants detected by multivariate tests. These tools contain a number of approved drug labels (including FDA- and EMA- approved drugs), treatment guidelines (including NCCN, ESMO) and clinical trials with global recruiting locations across different solid tumor types [64 65].
However, in a routine clinical setting, to avoid conflicting interpretations, only clearly defined and actionable pathogenic aberrations should be reported.

Could off-label/off-target gene alteration be of value for the patients?

We must continue to acknowledge and understand the vast, complex genomic variability of cancer in order to provide individuals with every opportunity for improved outcomes. The National Comprehensive Cancer Network (NCCN) guidelines for NSCLC [65] strongly endorse the use of broad molecular profiling to detect certain rare mutations using multiplex or NGS. The guidelines specifically report that "*EGFR* and *ALK* testing be conducted as part of broad molecular profiling". The NCCN Panel states that such testing would ensure that patients receive the most effective available targeted treatment for NSCLC [65].
The main practical problem for the oncologist in face of a patient with a rare but actionable mutation is how and where to treat him considering the strict regulatory rules of the National Health Care System.
Phase 1 and 2 clinical trials may not be running in all the Cancer Centers and off-label treatments requires specific consents and monitoring of sometime unknown side-effects. Recent development of information technology however will allow to have an updated catalog of the ongoing trials onboard of some of the new NGS platforms. However, it can be difficult insert a patient in a clinical trial running abroad or far from where the patients live.
Broad molecular profiling in solid tumors is at the beginning of its history. There is insufficient published evidence to support its use in all solid tumors. As for any assay analytical validity, clinical validity, and clinical utility must be proven.

Which is the appropriate turn-around-time (TAT) for lung cancer biomarker testing?

In general, the TAT should not exceed the following range: (i) for immunohistochemistry three working days, (ii) for FISH four working days, (iii) for mutational analysis seven working days. In case of cytological specimens, removal of the cover slip in xylene may require another one or two days, or even more.
The algorithmic approach used in the different institutions for the molecular target analyses may greatly influence the cumulative TAT, as FISH is frequently done after a preliminary IHC assay, and in some cases, in situ analyses are done following the results obtained by multitarget sequencing (e.g., *ALK* and *ROS1* are sometimes investigated only in *EGFR* and *K-RAS* wild type tumours).

Who should perform molecular testing?

In Italy, not all pathology departments are equipped to run molecular testing and a greater number of pathologists refers to outside molecular pathology laboratories. When determining the centre to select for outside molecular testing, the pathologist should ensure that the laboratory is accredited either at the national or at the international level. It is also relevant that the laboratory staff would include anatomic pathology-certified pathologists who verify specimen quantity and quality and supervise specimen selection, interface with clinicians, and troubleshoot problems. Processes should be established to ensure that specimens with a final morphological diagnosis are sent to external molecular pathology laboratories within three working days of receiving requests.

The liquid biopsy

When should we use the liquid biopsy?

The liquid biopsy may allow for the detection of activating and resistant *EGFR* mutations, in different clinical settings [66-71]. In particular, this assay, based on plasma derived circulating tumor DNA (ctDNA), has recently been implemented in clinical practice to select NSCLC patients for the first line treatment by tyrosine kinase inhibitors (TKIs) in which tissue samples were inadequate/unavailable for molecular analyses. Thus, the liquid biopsy enables for treatment decision making in a significant subset of patients, previously excluded from TKIs administration, due to the unfeasibility of tissue based assays. Liquid biopsy represents, furthermore, even a more appealing alternative to tissue samples for the detection of subclones resistant to first and second generations TKIs treatment, taking into account the difficulty and the risk to obtain a new tissue specimens (re-biopsy) and the heterogeneity of these mutations (e.g. *EGFR* T790M), especially in patients with widespread diseases [66 69-71].

Which markers can be investigated by the liquid biopsy?

The liquid biopsy can be used to identify *EGFR* mutations [66 69-71]. To date, there is the requirement to test for all individual mutations that have been reported with a frequency of at least 1% of *EGFR*-mutated lung adenocarcinomas. Thus, *EGFR* gene testing needs a large reference range including exons 18, 19, 20 and 21 to select NSCLC patients for appropriate TKIs treatment. As a general rule, the activating "sensitizing" mutations in exons 18, 19 and 21 allow for first (gefitinib and er-

lotinib) and second (afatinib) generation TKIs administration. Conversely, the analysis of *EGFR* exon 20 is important to detect the occurrence of acquired resistance (AR). In particular, the most relevant mechanism of AR relates to the emergence of an additional EGFR tyrosine kinase domain mutation, T790M, caused by a single base substitution, C to T, at nucleotide 2369; this mutation is found as a second mutation on the EGFR allele harboring the initial "sensitizing" EGFR mutation. Because AR is, within the tumor cell population, a subclonal process, the detection of the subclone of tumor cells that harbor T790M by sensitive and reliable techniques is crucial in order to allow for these patients a treatment with a third generation TKI (osimertinib).

Which technological platforms can be used for the liquid biopsy?

The ctDNA represents a minimal fraction (< 0,5%) of the total cell free circulating DNA (cfDNA) [67-69]. Thus, a reliable technology should have a sensibility as high as possible, to avoid false-negative results, and an absolute specificity, to avoid any false positive result. Allele specific real time PCR or digital droplet PCR (ddPCR) are the most appropriate technologies that are used in routine for the detection of *EGFR* mutations in liquid biopsy. These technologies, using specific probes, allow the identification of known mutations, but do not cover the whole spectrum of *EGFR* alterations, missing the identification of less common but clinically relevant mutations. In addition, their multiplexing power is restricted. These issues can be overcome by next generation sequencing (NGS) technology based on massive parallel sequencing of millions of different DNA molecules [68], which may allow for the detection of multiple mutations in multiple genes, whose application to the liquid biopsy is being currently pursued.

Is there a relation between ctDNA levels and tumour burden and stage?

There is great variability in the amount of ctDNA. Some authors have shown that ctDNA levels were positively associated with tumor stage and type of spread [72]. Thus, patients with higher tumor load might have more intensive tumor DNA released to the blood and ctDNA levels may reflect the tumor burden. However, as in many patients a minimal fraction of ctDNA is detectable, a reliable technology with the highest possible sensibility should be set-up. On the other hand, extremely sensitive procedures should be excluded to avoid false positive results.

The molecular pathology report

The referral process, an integral part of the diagnostic procedure, is the result of a multiple-step process that converts the results of a scientific analysis into useful clinical information for proper therapy implementation. The report should be compiled in a predisposed form, signed by the surgical pathologist and by the molecular pathologist and preferably structured in three main stages:

1. patient identification (patient's personal details; physician and/or structure that has requested the analysis) and type of material used (e.g. paraffin embedding, tissue section or cytological sampling), with reference to histological diagnosis;
2. result of the Molecular Test. According to the type of tests required (whether mutational or in situ tests), the information to be included in the report is the following:
   - *a. mutational test*:
     - the adequacy of the sample based on the number and percentage of neoplastic cells relative to the area of the biological sample selected for analysis;
     - the methodology and the commercial test used to perform the analysis and the analytical sensitivity of the method;
     - the exons submitted to analysis or the mutations investigated in the case of molecular-targeted methods;
     - the results of the tests expressed in terms of absence or presence of the mutation; in the latter case the mutation at DNA and protein level should be described, according to the international nomenclature;
     - in the case of material unsuitable for analysis, the reason for its inappropriateness should be reported.
   - *b. in situ analysis:*
     - the adequacy of the sample based on the number and percentage of neoplastic cells relative to the area of the biological sample selected for analysis;
     - the procedure used for analysis (FISH and/or IHC), with particular reference to the type of probe and to the manufacturer for FISH and to the clone and detection system for IHC;
     - the results of the FISH test must be expressed in terms of absence or presence of the rearrangement and in the latter, case the percentage of nuclei rearranged on the total number of nuclei submitted to analysis should be indicated. For immunohistochemical analysis the result should be expressed or through a binary (positive/negative) evaluation, or by a suitable score system with the possible addition of the percentage of positive cells, type of immunoreactivity (membrane or cytoplasmic) localization and of colour intensity;
     - in the case of material unsuitable for analysis, the reason for its inappropriateness should be reported.
   - *c. predictive analysis of multiple markers:* if the analysis has been performed by multi-gene methodology only to the markers specifically requested by the clinician should be reported.

However, some gene alterations could be included in the report, since this information are very helpful in order to screen and identify patients for enrollment in clinical trials (e.g., *BRAF* mutations, *c-MET* mutation).

3. Clinical Interpretation.
   The result of molecular analysis can be related to the appropriate drug treatment, on the basis of the data available in the literature.

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