



# The challenges of evaluating predictive biomarkers using small biopsy tissue samples and liquid biopsies from non-small cell lung cancer patients

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**Abstract:** The list of theranostic biomarkers for the care of patients with advanced stage or metastatic non-small cell lung cancer has lengthened considerably these last few years. Moreover, the advances in therapeutics will certainly increase the number and complexity of these tests performed in laboratories in the near future. In addition, the methods for investigation of biomarkers that require access to biological tissue are less and less invasive with the consequential increase in the use of small-sized tissue biopsies and cytological and blood samples. Thus, each laboratory must master the management of the biological samples according to the number and type of tests to be performed. This review will provide an update of the difference challenges facing pathologists and biologist in responding to the issues related to new treatments. These challenges concern: (I) the management of the pre-analytical phase; (II) the appropriate choice of the technological approach; (III) the performance of quality control; (IV) the mastering of the delay in obtaining the results; and (V) the economic model of the laboratory.

**Keywords:** Therapeutic strategy; biological samples; theranostic biomarkers; innovation; challenge

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## Introduction

Due to the discovery of molecules that are effective when based on the expression of proteins or genomic alterations, the therapeutic strategy for care of patients presenting with lung carcinoma is constantly moving (1,2). Targeted therapy, immunotherapy and/or chemotherapy are proposed to patients with advanced stage or metastatic non-small cell lung carcinoma, according to the detection on the tumors and/or in the blood of biomarkers of interest. The identification of these biomarkers predictive of therapeutic response is done with biological tests, the number of which has kept on increasing in these last few years (3). Thus, the prescription in routine practice of a treatment for a patient with a lung carcinoma is linked to the systematic investigation of a certain number of these biomarkers.

With the increase in the number of biological tests, the exploratory methods that provide biological material have evolved toward less and less invasive approaches (4-7). Thus, due notably to guided ultrasound approaches, endoscopic techniques have progressively focused on obtaining cytological samples (8). The use of flexible endoscopes of reduced caliber that allow access to distal tumors leads that the tissue biopsy samples have become smaller in size (9). The possibility of detecting genomic alterations with circulating free plasma nucleic acids has led to the use of liquid biopsies as a complementary and sometimes alternative approach to tissue biopsies (4,5,10,11).

Recently, the management of lung cancer patients has evolved within a new context that takes into account the increased number of clinical trials, the results that are

more and more promising and the need to rapidly validate diagnostic biomarkers, especially predictive ones, in routine practice. The increase in the number of theranostic biomarkers that have to be investigated first requires mastering the use of reliable diagnostics, while taking into account the available biological material. To confront these changes in practice the pathologist must face new challenges: (I) master perfectly the pre-analytical circuit of samples so as to preserve as much as possible the quality of the proteins and nucleic acids for the different analyses; (II) use both sensitive and specific techniques that are validated by external evaluation of quality in order to accredit the biological tests; (III) integrate into the circuit of analyses requirements concerning the delay in obtaining results, notably in the case of the need of an urgent therapy and according to the condition of the patient; and (IV) take into consideration the economic model of the laboratory and the institute, as well as the reimbursement of the costs of tests (12). In this context, since the algorithms associated with the different tests must be adapted to the available biological material and, finally for each patient, it is possible beside the “personalized medicine taking care of the patient”, to talk about of a “personalized taking care of the sample”.

This review presents a summary of the major challenges a pathologist must face to take optimal care of patients with non-small cell lung carcinoma, while considering the ever-increasing number of biomarkers, in particular for theranostic approaches, and the quantity, the quality and the origin of the available sample(s) for doing the tests.

### **The increase in theranostic biomarkers for personalized treatment of lung cancer: mandatory biomarkers and biomarkers of the future**

The international recommendations for care of patients with advanced stage or metastatic non-small cell lung cancer require that a number of biological tests be performed systematically (13). To date, the list of mandatory theranostic biomarkers includes investigation into genomic alterations in *EGFR* and *BRAF* (mutations), *ALK* and *ROS1* (rearrangements), as well as the immunohistochemical (IHC) expression of PD-L1 in tumor cells (13). In the USA these tests are companion diagnostics and must be performed according to the criteria and procedures approved by the Food and Drug Administration (FDA). In Europe, these analyses must be performed with tests

“validated” according to the European Medical Agency (EMA). Investigation into other molecular anomalies on other genes is not systematic in all centers and their detection can lead to inclusion of patients into clinical trials. The list of these additional genes of interest include *MET* (mutation and amplification), *HER2* (mutation), *RET* (rearrangement) and *NTRK* (rearrangement). Investigation into mutations on other genes (as *PI3KA* or *KRAS*) may be performed even if no therapeutic target has yet been identified. A promising predictive biomarker of response to immunotherapy was recently used during in clinical trials, the tumor mutational burden (TMB) (14). This molecular test is being clinically validated and may be rapidly added to the first list of mandatory tests. Other tests must be validated with a large cohort of patients before potential use in clinical practice. As an example, a mutation in *STK11* associated to mutation in *KRAS* seems to be a robust biomarker to predict resistance to immunotherapy (15). Beside these genomic biomarkers combined investigation into the *in situ* expression of tissue biomarkers, such as the number of intra-tumor CD8 positive lymphocytes associated with the percentage of PD-L1 positive tumor cells may predict the response to immunotherapy (16,17). There exist a substantial number of phenotypic biomarkers of potential interest and so several analyses must be performed to reinforce the predictive nature of the *in situ* approach. Antibodies to LAG-3, OX40, TIGIT, TIM3, VISTA, Foxp3, CD33, CD14, CD15 or IDO can be combined in a more or less comprehensive way (16,18). Other markers such as TCR or interferon gamma are being investigated too (16,17).

In the future, many molecular biomarkers will have to be combined. Then, the setup of adapted analytical statistical tools and of a number of validated clinical trials will be required for these combined approaches. Aside from these biomarkers, therapeutic combinations of immunotherapy associated with or not targeted therapies, or even chemotherapy, will lead to particularly complex predictive analyses.

### **“Genomic” biomarkers: present challenges**

The analysis of some molecular biomarkers requires extraction of nucleic acids from tissues, cells and/or blood samples. This extraction from tissue samples is almost always done with samples fixed in formalin. The reliability of the results correlates with the quality as well as the amount of the nucleic acid (19). This is particularly

true for analyses with tumor RNA. The quality and the quantity of nucleic acids varies according to the volume of the tissue sample, the percentage of tumor and non-tumor cells (in particular inflammatory cells) present on the surface for analysis and the zones of necrosis and/or hypoxia. Depending on the sensitivity of the method of analysis the results must take into account all these parameters. In particular, interpretation of a negative result must be made while considering the quantity and quality of the nucleic acids. Some approaches use the next-generation sequencing (NGS) technique, which requires a minimal amount of DNA that varies according to the sequencer (in general from 10 to 100 ng). This is particularly important when the tissue biopsy is of very small size or if the analysis uses circulating free DNA from blood samples. The possibility of integrating the evaluation of the TMB and the detection of activating or resistance mutations at a single time using one panel leads to a decrease in the delay and reduces the amount of DNA required. Cytological samples can be prepared in different ways, but ideally in cell blocks of a cell pellet fixed in formalin and included in paraffin. After control of the morphology of the tumor material in tissue sections, zones of interest can be selected and the nucleic acid extracted. Other types of preparation include cytological smears or pellets on slides after cytocentrifugation. The molecular analysis of the tumor cells thus isolated is possible but depending on the number of cells and the type of fixative used, since the quality and quantity of the DNA and RNA does not always provide satisfactory results. Liquid biopsies can be used to detect genomic alteration in free nucleic acids extracted from plasma (4,20,21). In this context, the amount of circulating tumor nucleic acids varies from one tumor to another one (4). Moreover, the quality of these nucleic acids in blood samples, in particular RNA, can also vary. Thus, the possibility of performing some methods that require an optimal quantity and/or quality of nucleic acids is sometimes uncertain. Moreover, the ratio of nucleic acids of somatic or tumor origin and of germ line origin leads sometimes to uncertain negative results. The importance of the pre-analytical steps must be highlighted (4,22). Most studies concern patients hospitalized in the same institute as the laboratory performing the molecular tests. This holds the strong advantage of allowing better standardization of the preparation of blood samples (using the recommended buffers and tubes for the analysis, reducing the delay for transfer of the sample, adapting the prescription and optimizing communication between the

different investigators). This is more difficult for centers receiving samples for analysis sent from distant sites. The analytical results must take into account the delay of transport and the type of blood collection tubes used, with the risk of increasing the amount of plasma germ line DNA as a result of lysis of hematological cells. Liquid biopsies hold the advantage of being able to be repeated in the case of an uncertain result and to monitor patients. Monitoring provides prognostic biomarkers (in particular as a function of the variability in the amount of plasma DNA) and detects early emergence of genomic alterations that suggest therapeutic resistance (for example, the emergence of the T790M and C797S mutations in *EGFR* or mutations in *ALK*) (4).

The methods for detection of theranostic biomarkers vary. They may concern targeted detection on a single gene (such as mutations in *EGFR* and *BRAF*, or rearrangements or mutations in *ALK* or *ROS1*), or detection with a panel of genes of variable size (23-25). Approaches such as NGS broaden investigations into an increased number of targeted molecules (such as for *RET*, *MET*, *HER2*, *NTKR*) but the results are only used if the patient can be quickly included into a clinical trial. Many challenges must be faced: (I) respond to needs of the clinician, depending on the available sample and the management of the pre-analytical steps; (II) use analytical methods and panels of genes adapted to the demands of the clinician; (III) obtain the results within a delay that allows targeted treatment; and (IV) ensure the quality of the different steps and set up regular external controls of quality, allowing accredited tests to be performed according to the recognized international requirements of quality.

### ***In situ* tissue and cytological biomarkers: the major present and future challenges**

The smaller and smaller size of tissue biopsies limits the number of tissue sections for IHC and *in situ* hybridization tests (26-31). If the architecture on bronchial micro biopsies is strongly suggestive of an adenocarcinoma or a squamous cell carcinoma of the lung it is important to not look systematically for certain markers such as TTF1, napsin A, P40, CK5-6 and/or for neuroendocrine markers (chromogranin, synaptophysin and CD56). Histochemical staining (PAS, Alcian blue) for detecting the presence of mucus must be then avoided depending on the case, in order to economize the amount of tumor material for other tests. Thus, the choice of the diagnostic tests depends

on the size of the biopsy and on the absence/presence of histological differentiation. Because of investigations into multiple target therapies the practices of pathologists are changing rapidly (27,32). The number of tissue sections (ideally 3 microns of thickness) varies according to the size of the bronchial or transthoracic biopsies. Aside from hematoxylin-eosin staining for evaluation of the morphology it is essential in the case of an adenocarcinoma or non-differentiated carcinoma to perform ALK and ROS1 IHC and to obtain two sections for fluorescence *in situ* hybridization (FISH) analysis of *ALK* and *ROS1* status assessment if necessary (33). FISH analysis should be also considered in the context of micro biopsies. It is not recommended to control a strong positive ALK IHC by ALK FISH (33). The *ROS1* status must be confirmed by FISH if the ROS1 IHC is positive (33). However, interest in FISH for detection of these biomarkers is limited if less than 100 tumor cells are present, where the threshold for positivity is set at more than 15% of tumor cells showing a rearrangement. At the same time, it is important to look for mutations in *EGFR* on one, two or three sections of variable thickness (5 to 10 microns), depending on the molecular biology method envisaged. Knowing that the incidence of mutations in *BRAF* in lung adenocarcinomas is low it is possible to wait for the results of analysis of *ALK*, *ROS1* and *EGFR* before looking for a *BRAFV600E* mutation (34). IHC with specific anti-BRAFV600E (clone VE1) antibodies can be performed, notably if a few tumor cells are visible (34). In fact, if a few positive tumor cells for VE1 are detected the patient can benefit from treatment targeting the *BRAFV600E* mutation (34). The presence of a carcinomatous lymphangitis with detection of a few tumor cells in vessels or of rare clumps of carcinomatous cells in the stroma is a factor limiting molecular biology exploration. It is notably difficult to obtain a sufficient amount of DNA for detection of mutations in *EGFR*. In this case, and exceptionally, it is possible to use antibodies that evaluate indirectly the status of *EGFR* (del19 and L858R) (35,36). However, this approach is not recommended at the international level (13).

The possibility of administering first-line immunotherapy to patients with non-small cell lung carcinoma, in the absence of the *ALK*, *ROS1*, *EGFR* and *BRAF* genomic alterations, and the development of anti-PD-L1 IHC has drastically changed the work of the pathologist (37). The size of the biopsy is a major criterion to take into consideration when performing PD-L1 IHC. In fact, the threshold for positivity for the administration

of first-line immunotherapy is 50% labeled tumor cells and evaluation must be done with at least 100 tumor cells. PD-L1 shows heterogeneous tissue expression (38-44). Most of the studies on this subject show discordance between the PD-L1 status evaluated with biopsies and with surgical specimens taken from the same patients (38-44). Thus small-sized biopsies may not be representative of the overall status of the tumor mass and PD-L1 status may be underestimated in these biopsies (40). PD-L1 IHC should be performed on one of the first tissue sections for evaluation with the largest tumor surface and before tissue material "exhaustion". The development of immunoncology and of new treatments in this domain could be associated in the near future with IHC for molecules other than PD-L1. The complexity of these tests could limit evaluation on small-sized samples. Evaluation of several molecules of interest at the same time on a low number of tumor cells and of the microenvironment raises two questions: (I) the biological relevance of considering results obtained from tumors with heterogeneous expression; and (II) the difficulty of obtaining multiple tissue sections from small-sized tissue samples.

Cytological samples can also be used to evaluate the different above-mentioned biomarkers (45). Depending on the pre-analytical phase, the technique of immunocytochemistry (ICC) cannot be standardized and the results cannot be similar in quality to those obtained after fixation in formalin (45). In the absence of independent clinical validation associated with clinical trials, ICC for PD-L1, ALK or ROS1 are difficult to be considered as companion diagnostic tests. Particularly, analyses integrating the expression of PD-L1 on cytological samples should be considered very carefully (46-51). The expression of biomarkers on inflammatory cells should be evaluated with caution since, depending on the specimen and the site, these cells may not be representative of cells of the tumor microenvironment and correspond to cells in blood and/or cells of lymph nodes. Thus, analyses of immunological markers with immune cells in cytological samples must take into consideration this context.

### Which algorithm to propose?

Depending on the volume and the quantity and nature of the sample sent to the laboratory, the situation may be ideal for detection of theranostic biomarkers or may require making choices and establishing priorities with regard to the clinical examination to be performed. In real

life the situations encountered are not those reported in certain publications, in particular those of clinical trials. In fact a number of hurdles that depend on the transfer of samples, the hospital organization, the degree of expertise of the medical and technical staff and the possibility of methodological development of the laboratory can be encountered.

To date, two principal algorithms can be considered for the handling of tissue biopsies of good quantity (in particular with a percentage of tumor cells above 20%), depending on the histological type (adenocarcinoma, squamous cell carcinoma, non-small cell carcinoma of “non-determined” diagnosis) on hematoxylin-eosin stained sections. In the case of an adenocarcinoma the first algorithm consists in analyzing the expression of PD-L1 by IHC and then extracting DNA for NGS with a panel of genes of interest. The obvious choice of these two tests holds the advantage of detecting all the therapeutic targets associated with the present recommendations (PD-L1, *EGFR*, *ALK*, *ROS1* and *BRAF*), but at the same time some other potential targets (on the *MET*, *RET*, *HER2*, or *NTRK* genes for example) which can be associated with clinical trials. However, this approach has its limits: it requires an adapted infrastructure and expertise, can increase the turnaround time in obtaining the results, depending on the level of organization, and is probably more costly, according to the panel used. The second algorithm consists in not performing straight away NGS and to use this approach only if the tumor shows no genetic alteration in *EGFR*, *ALK*, *ROS1* or *BRAF* and if the expression of PD-L1 on the tumor cells is lower than 50%. In this context, IHC is performed systematically with anti-PD-L1, anti-*ALK* and anti-*ROS1* antibodies and then mutations in *EGFR* are looked for using a targeted molecular biology test. IHC with the clone VE1 or *BRAF* targeted molecular analyses can be used to evaluate the *BRAF* status (34). Some pathologists wait for the *EGFR*, *ALK*, and *ROS1* status to be known before deciding if it is necessary or not to look for a *BRAF* mutation and also consider NGS to cover more genomic alterations. This algorithm has the advantage of rapidly giving the results of the *ALK* and *EGFR* status. Positive IHC for *ROS1* leads to an additional delay due to FISH analysis for *ROS1*. The costs of the analyses for this algorithm depend on the different antibodies and methods used.

When the amount of tumor tissue is limiting (small-sized tissue biopsies, necrotic zones, low percentage of tumor cells), it is possible to adapt the previous algorithm. IHC (*ALK*, *ROS1*, *BRAF*) has the advantage of being able

to visualize on a small number of tumor cells the presence of target molecules. For the analysis of *EGFR* mutations, the minimal amount of DNA depends on the sensitivity of the method used. However, if only a very few tumor cells are visible (for example in case of a carcinomatous lymphangitis) this technique does not allow evaluation of the *EGFR* status and an IHC with antibodies to mutated *EGFR* can be considered.

When not enough or no tissue or cytological material is available for molecular analyses (a patient in a too poor condition for an invasive approach, a tumor not accessible to obtaining a biopsy, a non-contributory biopsy), a liquid biopsy can be performed to look for genomic alterations (4,10). Beside *EGFR* assessment, evaluation of the PD-L1 status has not been clinically validated with liquid biopsies and the sensitivity of detection of rearrangements in *ALK* and *ROS1* is open to discussion depending on the method of analyses (20,52,53). In the case of an accessible tumor that gave a non-contributory result an additional tissue biopsy must be proposed (53).

### The key points to master and what are the perspectives?

“Do more with less” is the direction to take for detection of biomarkers on lung cancer patient samples collected using less and less invasive acts. This has put new constraints on pathologists. It is difficult in most of the time and in “the real life” to combine all the techniques using the biological material sent to the laboratory. The heterogeneity of tumors complicates the phenotypic and genotypic analyses performed with small tissue specimens (54-58). This is particularly true for multiplex IHC or for NGS approaches that use broad panels (59,60). The tumor heterogeneity is also a dynamic phenomenon and may complicate the assessment of certain biomarkers during the follow up of lung cancer patients (58,61). Tests performed in an automatic fashion can give rapid results. However, the cost of systematically performing certain analyses such as NGS are considerable compared to the level of care proposed as a function of the results. Aside from chemotherapy, the two major therapeutic opportunities are immunotherapy and therapies targeting molecular anomalies in *EGFR*, *ALK*, *ROS1* and *BRAF*. Thus, the discovery of other molecular targets by NGS rarely leads to therapeutic propositions and finally to inclusion into clinical trials. Moreover, these clinical trials are not available at all hospitals and may require the patient to go rapidly to a distant location from

its home to be treated. The multiplication and complexity of the tests has led to question the need for centralization of expert centers with the necessary platforms and competence. So, is it still possible to perform the majority of the tests in the majority of public and private laboratories close to patients and clinicians? Moreover, irrespective of the level of organization, investigations into biomarkers must adhere to the requirements for quality set out by international norms and to, even better, receive accreditation (62).

The molecular therapeutics that target either proteins (immunotherapies) or genomics (targeted therapies) anomalies and the tests for detection that require combined morphological and molecular analyses necessitate different expertise (morphologists and molecular biologists). To provide therapeutic solutions to patients in the future the analytical techniques will become even more complicated, associating evaluation of several morphological signals using quantification software and complete study of the exome. In this context, deep learning and machine learning may probably provide rapid guidance in making therapeutic decisions (63).

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