



# Tumor mutational burden in non-small cell lung cancer—the pathologist's point of view

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**Abstract:** In non-small cell lung cancer (NSCLC), the pathologist has contributed to the development of personalized medicine from the determination of the right histological type to *EGFR* and *ALK/ROS1* molecular screening for targeted therapies. With the development of immunotherapies, pathologists intervene forefront with programmed death-ligand 1 (PD-L1) immunohistochemical testing, companion test for pembrolizumab monotherapy, first line and complementary test to the other programmed cell death-1 (PD-1) PD-L1 inhibitors. Recently, tumor mutational burden has emerged as a promising tool to evaluate sensitivity to immunotherapy (IO). The pathologist has a crucial role in the setting of tumor mutational burden (TMB) testing for the selection and the preparation of the sample for high throughput molecular analysis, and in the first steps of the next-generation sequencing (NGS) workflow.

**Keywords:** Non-small cell lung cancer (NSCLC); biomarkers; immunotherapy (IO); tumor mutational load; preanalytical; quality assurance

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The clinical breakthroughs of immunotherapy (IO) and personalized medicine have modified the survival and the quality of life of many cancer patients. These advances are possible through the development of predictive biomarkers of response to treatments. By using our own immune system to fight cancer, IO is also revolutionizing our concept of biomarkers (1). Thus, many emergent predictive biomarkers for cancer IO derive not only from the tumor cells, but also from their microenvironment, and from our innate capacity to develop an immune response, including major complex of histocompatibility and microbioma. The concept of a cancer immunogram combining different markers is emerging (2). The pathologist has a pivotal role in this quest for biomarkers in the setting of IO. Among the myriad of candidates, tumor mutational load or TMB is emerging as the new kid on the block (3).

## Pathologist and non-small cell lung cancer (NSCLC)

### Diagnosis

In lung cancer, the pathologist has contributed to the development of personalized medicine, starting with the determination of the right histologic subtype (problem solved by simple immunohistochemistry: TTF1, p40, CK5/6, neuroendocrine biomarkers) (4). This guide chemotherapy regimens for non-small cell and non-neuroendocrine carcinoma: i.e., gemzar, platinum salts and contraindication of anti-angiogenic therapy for squamous cell carcinoma (SCC); pemetrexed, platinum salts for non-squamous. Moreover, histologic subtypes determination allows triage for molecular testing in NSCLC; SCC being excluded from molecular screening in the context of targeted therapies, so far (except for SCC developed non-smokers) (5).

### Markers for IO

The level programmed death-ligand 1 (PD-L1) expression detected by immunohistochemistry, on the surface of tumor cells, is so far, the unique validated biomarker for checkpoint inhibitors therapies anti programmed cell death-1 (PD-1) or PD-L1 in lung cancer (5-7). Although PD-L1 expression alone is often insufficient for patient selection in several tumor types. Recently, new insights have focused the important role of the tumor mutational load in this setting (8,9). The tumor genome is a driver of anti-cancer immunity. Depending on the tumor mutational burden (TMB), the response to IO varies, suggesting that neoantigens generated by these mutations are crucial targets for T cells in cancer immunity (10). Today, TMB stands as one of the available tools to evaluate sensitivity to IO (3).

TMB accounts for the number of non-synonymous mutations per megabase. TMB is influenced by different factors including microsatellite instability, somatic mutations in POLE and POLD genes, exposition to tobacco, alkylating agents or sun, leading to a highly abnormal level of mutations in tumor DNA (11-14).

### Pathologist and TMB: genotype/phenotype associations?

The first implication of the pathologist will be to identify tumors that can harbor a high mutation rate, such as tumors with a deficient mismatch repair (dMMR) pathway, or an hypermutator phenotype. Some pathological features such as poor differentiation, massive lymphocytic infiltration is more frequently observed in the context of dMMR, POLE exonuclease domain alterations (14-16) and are associated to high TMB (12). Yet, the reverse is not always true and for instance, melanoma, SCC, lung cancer frequently has a high TMB but are exceptionally dMMR (<1%) or POLE deficient (~3%) (13,14,16,17).

The clinical history such as in colorectal cancer young age, right colon localization, is also informative for a potential dMMR phenotype, but no such clinical association exists in NSCLC where dMMR is found in 0.8% of lung adenocarcinoma (18). The quantification of tumor infiltrating lymphocytes or tumor-infiltrating lymphocytes (TILs) on haematoxylin and eosin (H&E) slides has the potential to become a new tumor biomarker, being an easy approach to evaluate the immunogenicity of a tumor. The international TILs Guidelines provide standardization for a visual evaluation of H&E sections by pathologists in lung

cancer (19). Their robustness has been demonstrated in international ring trials. Nevertheless, despite promising observations, linking the level of TILs and response to IO, more clinical validation is required to confirm TILs as strong predictive biomarkers for IO in lung cancer (20). In summary, the pathologist evaluating a tumor can give some valuable biomarkers for IO prediction such as PD-L1 status, TILs count, dMMR status. However, those markers are neither exclusive nor 100% sensitive, but rather complementary of other biomarkers such as TMB, or composition of the microenvironment.

TMB is not standardized so far, but the techniques to explore it are complex and require the use of next generation sequencing. When implementing TMB measurement assays, it is important to consider factors that may affect the method workflow, the results of the test, and the interpretation of the data. Such factors include the type of specimen analyzed, sample quantity and quality, sequencing platform, genome coverage, bioinformatics pipeline, and the choice of the thresholds that determine a high TMB. The different techniques and their interpretation tools are detailed elsewhere in this special issue.

The pathologist has a crucial role in this setting for the selection and the preparation of the sample for high throughput molecular analysis, and in the first steps of the next-generation sequencing (NGS) workflow.

### Tissue samples

Clinical samples for NSCLC diagnosis are heterogeneous. To ensure proper diagnosis and enough material for subsequent analysis, coordination between clinicians and pathologists is necessary. Different groups have published guidelines for clinicians concerning the number of samples required, to maximize the volume of tissue for diagnosis and subsequent analysis (21). At least five endobronchial/transbronchial forceps biopsies ± five additional, at least four EBUS aspirations per target, at least two percutaneous core needle biopsies with additional three to six are required for proper diagnosis and molecular testing. Cytoblocks have also recently being validated for IHC, FISH and molecular screening with small panels of genes. Surgical specimens are handled following codified procedures; collection of frozen material is optional. Three to five tumor sections (5–10 mm thick) are sampled from the surgical specimen, after proper fixation (22).

As the amount of tissue is often limited, different strategies can be developed such as sample prioritization to

spare tissue and allow the realization of all the analysis for accurate histological diagnosis (if needed) and, then, for the study of predictive biomarkers i.e., PD-L1 for all, and for non-SCC or SCC non-smokers, preservation of four to six blank sections for Alk, Ros1 by immunohistochemistry (and validation by fluorescent in situ hybridisation (FISH) under some circumstances) and molecular screening for mutations (EGFR at least, but usually KRAS, BRAF, HER2, ...) (21,23). The management of the blocks has to be entrusted to qualified technicians.

Samples used for TMB assessment may be formalin-fixed, paraffin-embedded (FFPE) tumor tissue, cytology samples or liquid biopsies. FFPE samples are the most commonly available for clinical testing, as the practise of systematic storage of frozen-specimens is not developed, especially for biopsies and in community centers. While some evidence exists for the feasibility of using cytology and fine-needle aspiration (FNA) samples for the analysis of small panels by NGS (24), these samples can yield variable amounts of DNA that may not always be sufficient for in-depth molecular analysis (25,26). In summary, more than 70% of patients have an advanced disease. Additional tests are frequently required. Thus, cytology or small biopsy samples with a small number of tumour cells might only allow diagnosis and classification of tumor type (i.e., adenocarcinoma). Then additional molecular tests may be compromised, especially large panel or whole exome sequencing (WES). New biopsies can be ordered by the multidisciplinary tumor board (MTB). Liquid biopsies represent an option for the future for TMB evaluation (27).

#### **Quality of the samples—preanalytical considerations**

FFPE samples present a risk for use with high-throughput genomic assays because preanalytical factors common to NGS testing can affect the sample integrity or amount of DNA that can be extracted (26,28). An accurate molecular test requires a well-preserved specimen. The time of warm and cold ischemia should be as short as possible and can be monitored for a better traceability. Ischemia affects both protein and nucleic acids quality (29). The delay between tissue acquisition and fixation should be shorter than 30 minutes. It depends also upon the environmental temperature (23). The length of fixation depends on the size of the sample. Between 6–48 hours are recommended (12 optimal for a surgical specimen), in a sufficient quantity of fixative (30). Biopsies and cytology samples are fixed directly after the procedure, before the transfer to

pathology. Cell blocks after centrifugation and formalin fixation are the favourite method for processing cytological specimens. They are handled like histological specimens. For surgical specimen a vacuum preservation can avoid delay to fixation, if the transfer to the pathology lab and the gross examination are expected to be superior to 30 minutes. Immediately after removal, the sample is conserved by sealing under vacuum in special plastic bags and placing it in a controlled environment (4 °C) (21). The universally recommended fixative is 10% neutral buffered formalin (4% formaldehyde).

Formalin fixation can affect sample integrity (31). FFPE tissues exhibit a high frequency of non-reproducible sequence alteration because of cross-linking cytosine nucleotides on DNA strands. Taq-DNA polymerase fails to recognize the cytosine and incorporates an adenine in the place of a guanosine, creating an artificial C-T or G-A mutation (32). The error rate is around 12% but those alterations are random and non-reproducible (33). FFPE slides and extracted DNA have been shown to be reliable for TMB assessment for 6 months and likely beyond (34).

#### **Sample selection for TMB: quantity**

The pathologist plays an important role in the selection of the best sample for molecular analysis. This sample should be as pure as possible in invasive neoplastic cells. The cellularity of the sample is evaluated in a standardized and reproducible manner, following guidelines (35). Cellularity is expressed as percentage of total cells/nuclei. This estimation is of outmost importance in determining success or failure of subsequent testing, by defining the lower limit of detection and a minimum percentage of neoplastic cells present can be applied (30% for non NGS, 10% for NGS) (21,23,24). A macrodissection allows a greater tumor DNA yield for assessment (21,23,36). DNA quantity varies from 10–100 ng for gene panels and around 250 ng for WES (36). It is critical to inspect the last section after the slides cut from the selected material delivered for molecular testing. If possible, avoid providing samples with extensive necrosis, pigmentation, huge inflammation. If not possible, due to sample limitation, this should be emphasized in the report, along with cellularity.

#### **Pathologist implication in the workflow**

Turnaround time is influenced by the time required for different steps in the NGS workflow. As TMB assessment

is complex and long (run time and interpretation), there is increasing efforts to shorten the interval between the prescription of the molecular test by the oncologist and the time treatment is initiated, as this may influence prognosis. These upstream steps imply, surgeons, radiologists and pathologists (21,36). The workflow steps under the responsibility of the pathologist encompass sample acquisition, pathology review and diagnosis (two to four days). Then the patient file is presented to the MTB, actionable mutation screening is prescribed (one week). If the sample centralized, the local pathologist has to batch and send to the central lab (variable length). As TMB assays are not routinely performed, there is a possibility of a second batch and send for TMB to a specialized lab (two to three weeks).

In lung cancer, before the development of NGS, the turnaround time for actionable mutation screening, i.e., EGFR and Alk, was approximatively five working days after the reception of the sample by the platform. A variable difficult to influence is the transfer duration between a local pathology department and a central molecular platform. During the last 10 years, with education and under the influence of scientific societies, the transfer times are reduced to three to five days in general (37-39). To improve the timing of molecular tests including TMB, we have some opportunities: (I) reflex testing of “routine” predictive markers by the pathologist after the diagnosis of a metastatic NSCLC; and (II) democratization of TMB assays to streamline diagnostic testing for TMB alongside other genomic markers, avoiding a second run.

### Quality assurance

Quality assurance is requested at different steps of the TMB analysis. As previously mentioned this is a multidisciplinary shared concern. There is a need for all to be coordinated and well informed on the necessary approaches to achieve a reliable assay result. Molecular tumor boards have been created to guarantee global harmonization in tumor-sequencing practices (40). Concerning the pathologists, guidelines have been published to help the different structures (local or central) to develop standard operating procedures (SOPs) in order to harmonize practices and provide excellent quality nucleic acids (41). Efforts were developed to standardize the evaluation of tumor cellularity (35). It is important that these procedures be conducted in a standardized way. SOP as per ISO 15189 should be put in

place from specimen identification to the final reports (36). Recent NGS guidelines underline the role of the pathology laboratory director in using an error-based approach that can detect potential sources of errors that could happen throughout the analytical process (41).

### Conclusions

Validated biomarkers should accurately predict patient responses to IO. TMB assessment is emerging as a very promising factor. Using valuable lessons, learned from the implementation of targeted therapies in lung cancer, the pathologist has to play a key role in the development of TMB as a clinical diagnostic tool.

As the field evolves, reliable TMB assessments will be carried out in clinical practice, and included in the molecular workflow of lung cancer, as a reflex test. However, definitive recommendations may not be possible until more data are available from prospective clinical trials and external quality-control schemes to optimize the testing. Trained pathologists and technicians will provide high quality samples and efficient workflow to optimize advanced lung cancer patient’s management.

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