

# Integrating the molecular background of targeted therapy and immunotherapy in lung cancer: a way to explore the impact of mutational landscape on tumor immunogenicity

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**Abstract:** The results of randomized clinical trials employing immune checkpoint inhibitors for pre-treated advanced non-small-cell lung cancer (NSCLC) have recently revolutionised the standard available option for this disease setting. Nevertheless, the validation of reliable predictive biomarkers, able to define that proportion of patients most likely to benefit from immunotherapy, represents a crucial and still unsolved issue. This intensive research aimed at selecting potentially predictive biomarkers for immunotherapy is developed together with a wide range of analyses investigating the molecular profiling of lung cancer, leading to the spontaneous question of how these two parallel aspects of the same disease may coexist and influence one another. The potential impact of the mutational landscape of lung cancer on tumor immunogenicity (in both oncogene-addicted and molecularly unselected disease) will be explored and discussed in this review in order to begin to answer the unsolved questions.

**Keywords:** Immunotherapy; lung cancer; mutations; neoantigens; immunogenicity

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## Introduction

In recent years, technological advances in the study of biological background of tumors provided the proof-of-principle that non-small-cell lung cancer (NSCLC) is represented by a group of molecularly heterogeneous diseases. Several genetic mutations have been identified and validated as oncogenic drivers, able to determine the development and maintenance of specific subclasses of NSCLC (1).

The thrilling discovery is that several mutations are

‘actionable’, or rather targetable with specific drugs, radically transformed the care and prognostic perspectives of lung adenocarcinoma patients. The era of targeted therapy in lung cancer broke through with the discovery of driver mutations in the epidermal growth factor receptor (EGFR) (2,3). Several randomized clinical trials conducted in NSCLC carrying activating mutations of EGFR clearly demonstrated that tyrosine kinase inhibitors largely improve prognosis, disease control, symptoms and quality of life when compared to traditional platinum-based

chemotherapy (4,5). Other potentially targetable driver mutations have been identified in lung adenocarcinoma, including HER2, MET and fusion oncogenes involving anaplastic lymphoma kinase (ALK), ROS1 and RET (1,6).

The introduction of tumor genotyping into therapeutic decision-making, the discovery of new potential targets and the technological advances in multiplexed genotyping platforms, led to development of several large-scale screening programs to determine the true frequency of oncogenic drivers (7-9). The final aim is to provide practical routine molecular profiling techniques able to collect reliable information to guide treatment of patients and simplify studies with targeted agents (10).

Recently, the therapeutic opportunities of lung cancer patients further expanded with the introduction of immunotherapy. The great excitement among scientists, physicians and patients after the presentation of successful results in treating subsets of cancer patients quickly led to the onset of pressing questions regarding what parameters may predict response. The intensive research aimed to identify potentially predictive biomarkers for immunotherapy is developed together with the above-described investigations about the molecular profiling of lung cancer, leading to the spontaneous question of how these two parallel aspects of the same disease may coexist and influence one another.

### **Immunotherapy and predictive biomarkers**

The results of randomized clinical trials employing immune checkpoint inhibitors for pre-treated advanced NSCLC have recently revolutionised the standard available option for this disease setting, with significant advances for squamous histology and good perspectives for non-squamous (11,12). Nevertheless, while nivolumab demonstrated a significant improvement in terms of survival for squamous histology regardless of the immunohistochemistry expression of the programmed-death ligand-1 (PD-L1), the benefit of receiving this antibody in comparison with docetaxel seems to be higher in those patients with high tumoral PD-L1 expression in the case of non-squamous NSCLC (regardless of the cut-off, 1%, 5% or 10%) (11,12). The different impact of the candidate predictive biomarker PD-L1 according to histology is still debatable. In this regard, although the benefit of nivolumab seems to be restricted to those patients with high tumoral PD-L1 expression in the case of non-squamous NSCLC, the same correlation has not been observed in squamous histology. Thus, the

retrospective evaluation of PD-L1 expression in archival samples does not lead to definitive conclusions. Moreover, in the pivotal trial of pembrolizumab in NSCLC, although patients with squamous histology represented only a small proportion, the predictive effect of PD-L1 seems to be confirmed using contemporaneous samples (13). Therefore, the hypothesis that the impact of a rich cohort of coexisting mutations (as in the squamous subtype) may overcome the predictive power of PD-L1 must be validated.

The results of the Checkmate 057 are coherent with those of the randomized phase II POPLAR trial employing the anti-PD-L1 atezolizumab for all the histologies of NSCLC, although the immunoscore for biomarker positivity comprise both the expression on tumoral and tumor-infiltrating immune cells (14). Although the overall results about the predictive role of PD-L1 are convincing, still unsolved issues are represented by the determination of the best cut-off expression level and the different analytic techniques adopted across different trials. In this regard, advanced NSCLCs receiving pembrolizumab (an anti-PD-1 immune checkpoint inhibitor) are significantly much more likely to benefit from this drug if PD-L1 is strongly expressed upon tumor cells (>50%) (13).

Currently, the complexity of factors triggering the immune response to efficiently recognize and neutralize a specific antigen can not be easily simplified by the direct pharmacodynamics of an antibody binding PD-L1. As recently demonstrated, other immune mediators are potentially involved in atezolizumab-driven immune responses and only partially mediated by PD-L1 overexpression (15). In addition, patients with low PD-L1 expression may respond to an anti-PD-L1 antibody as well, underscoring the complexity of biological mechanisms supporting the immune response. PD-L1 may probably be considered just one of the predictive factors for immunotherapy and recent data suggest that the combination of other markers of immune cell infiltration (such as CD10 and CD20) and their ratios may have a prognostic (and maybe predictive) implication (16).

The expression of PD-L1 (regardless of the method), and all the other biomarkers of immune microenvironment are significantly affected by analytical and reproducibility limitations with important implication for clinical practice. Thus, the reported practical difficulties in interpreting the results of trials according to PD-L1 expression, strongly call for the identification and validation of biologically relevant and reliable biomarkers, determined with reproducible and harmonized assay procedures (17).

## Impact of mutational landscape on response to immunotherapy

The identification and validation of those factors able to determine tumor immunogenicity represents a major challenge for research in the immunotherapy field. The immunogenicity of a tumor depends on its antigenicity and a series of immunomodulatory factors produced both by tumor and host cells in the tumor microenvironment (18).

Tumor-specific antigens can be classified into two main categories: tumor-associated self-antigens (such as cancer-testis and differentiation antigens) and antigens derived from tumor specific mutant proteins [also called neoantigens or mutation-associated-neo-antigens (MANA)]. While T-cell reactivity against self-antigens is usually weak and characterized by a low avidity binding, neoantigens are fully human specific, and are therefore theoretically expected to induce a stronger immune response without toxicity against healthy tissues. The production of neoantigens is induced by a mutational event that may involve antigen expression as well as its processing and presentation to immune cells (19).

The finding that immune cell populations in tumor infiltrates may affect responsiveness to checkpoint inhibitors highlights the necessity to understand which antigens can induce an effective immune response against the tumor.

Some preliminary studies suggested that tumors with a high load of somatic mutations are more likely to respond to immunotherapy through the presentation of neoepitopes that may behave as neoantigens (20-22). To test this hypothesis, Snyder *et al.* performed whole-exome sequencing of tumor samples from melanoma patients treated with the anti-CTLA-4 specific antibodies ipilimumab and pembrolizumab. As expected, the high load of somatic mutations correlated with response to therapy in most patients, but surprisingly not in all. Computational analysis demonstrated that specific mutation-derived neoepitopes were shared by those patients responding to immunotherapy, defining a signature able to predict long-term clinical benefit from checkpoint blockade (23). In this regard, the quality of mutations, more than the quantity, may have the strongest predictive value (24). The identification of those mutations producing immunogenic neoantigens, able to trigger an effective immune response, is essential to the understanding and manipulation of T-cell response against cancer.

Available data support the fact that T-cell adaptive immune response might be preferentially directed towards

a specific subset of mutant sequences, facilitating the bioinformatic identification of possible neoantigens for therapeutic targeting (25). Yadav *et al.* developed, in the context of a murine tumor, an innovative approach that combines whole-exome and transcriptome sequencing analysis with mass spectrometry to identify neo-epitopes. Vaccination of mice confirmed the reliability of this approach, virtually applicable in any cancer cell type, with each predicted immunogenic peptide yielding therapeutically active T-cell responses. Interestingly, the identified neoantigens usually derived from proteins not directly related to tumorigenesis, enhancing the significant role of passenger mutations in the determination of cancer immunogenicity (26). Another pivotal study used genomic and bioinformatic approaches to rapidly and accurately identify tumor-specific mutant proteins, useful not only as targets of checkpoint inhibitors, but also as components of major histocompatibility complex (MHC) tetramers that can be used to identify tumor-specific T-cells as biomarkers of successful immune responses against cancer (27). In this regard, Kreiter *et al.* proposed a complex approach by integrating technological advances in the field of next-generation sequencing, computational immunology and synthetic genomics to explore the neoantigen repertoire in order to identify those that are most immunogenic (according to their expression level and MHC class II-binding capacity). Vaccination with synthetic poly-neoepitope messenger RNA vaccines, produced against these carefully selected neoantigens, induces tumor rejection of established growing tumors in mice models (28).

NSCLCs, particularly those related to the chronic exposure to carcinogens in cigarette smoke, are usually characterized by a high mutational burden, representing a biologically rationale target for immunotherapy approach (29). In this regard, the pivotal study of Rizvi *et al.* explored the potential influence of the NSCLC mutational landscape in determining sensitivity to PD-1 blockade (with pembrolizumab) (30). Whole exome sequencing, conducted in two independent cohorts, demonstrated that patients with high nonsynonymous mutation burden, compared with those with low mutation burden, experienced improved objective response rate (63% *vs.* 0%), progression-free survival (14.5 *vs.* 3.7 months) and durable clinical benefit (73% *vs.* 13%) from pembrolizumab. Efficacy was also correlated with molecular smoking signature, higher neoantigen burden and DNA repair pathways mutations (30).

Several studies reported that only a tiny fraction of neoantigens is predicted to bind to MHC molecules,

becoming effective targets of endogenous T-cell response. Nevertheless, from a purely probabilistic point of view, tumors with a high number of mutation-associated neoantigens are more likely to produce effective epitopes, stimulating the antitumor immune system reaction. This hypothesis supports the correlation between the high mutational load and the response rate observed with anti-CTLA-4 in melanoma and anti-PD-1 in lung cancer (23,30).

According to this hypothesis, even tumors with mismatch-repair deficiency (MRD) could represent potentially strongly immunogenic disease. In fact, MRD colorectal cancers have 10 or 100 times as many mutations as mismatch repair-proficient (MRP) cancers (31). Moreover, they are characterized by a prominent lymphocytes infiltrate supporting an effective immunogenic value (32,33). To validate this hypothesis, a phase II trial evaluating the clinical activity of pembrolizumab has been conducted in progressive metastatic carcinoma patients with or without MRD (34). Patients with MRD colorectal cancer demonstrated a clinical benefit of immune checkpoint blockade with pembrolizumab compared to those with MRP cancers, both in terms of immune-related response rate (40% *vs.* 0%) and of immune-related progression-free survival (78% *vs.* 11%). A statistically significant prolongation of median progression-free survival and overall survival favouring the cohort with MRD tumors was also reported. According to the available evidence, the high mutational load was associated with prolonged progression-free survival ( $P=0.02$ ). In this regard, with whole-exome sequencing analysis MRD tumors presented a mean of 1,782 mutations per tumor as compared with 73 in MRP tumors ( $P=0.007$ ) (34).

### Immunogenicity in oncogene-addicted disease

As previously discussed, several genetic mutations have been identified and validated as oncogenic drivers in NSCLC (1). This finding, in the context of immunotherapy research, implies intriguing questions regarding the interaction and mutual influence of the two pathways, particularly in terms of response to treatment (both with tyrosine kinase inhibitors and immunotherapeutic agents).

Besides the above-described limitations in terms of both analysis and interpretation, PD-L1 seems to be differentially expressed according to the molecular phenotype of tumors. In this regard, a recent analysis assessed PD-1/PD-L1 expression in NSCLC patients harboring EGFR mutations, ALK translocations or KRAS mutations (35).

Whereas PD-1 positivity was significantly associated with active smoking status ( $P=0.02$ ) and with the presence of KRAS mutations ( $P=0.006$ ), PD-L1 positivity correlated to adenocarcinoma histological subtype ( $P=0.005$ ) and EGFR mutations ( $P=0.001$ ). PD-L1 positivity was also associated with improved benefit from gefitinib and erlotinib in terms of response rate ( $P=0.01$ ), time to progression ( $P<0.0001$ ) and overall survival ( $P=0.09$ ). Interestingly, median PD-L1 levels were 5 times higher in ALK translocated tumors compared with triple negative, although the association was not statistically significant (35).

A growing body of evidence suggests that oncogenes may indirectly influence tumor microenvironment, regulating the release of ligands and cytokines (36). EGFR represents one of the most commonly mutated oncogenes in NSCLC patients (37). Preclinical studies conducted in murine melanoma models demonstrated that the activation of EGFR might suppress the immune response against cancer (38). Based on these findings, a pivotal study analysed the immune microenvironment and the immune-related pathways in EGFR-driven mouse lung tumors (39). A correlation between EGFR activation and a composed signature of immunosuppression (manifested by the upregulation of PD-1, PD-L1, CTLA-4 and several tumor-promoting inflammatory cytokines) was reported. This role of the EGFR pathway was independent of its traditional activity in cell proliferation and survival, suggesting an active involvement of EGFR as a modulator of tumor microenvironment. Concerning pharmacological inhibition, the tyrosine kinase inhibitors targeting EGFR reduced PD-L1 expression with a positive impact on mice survival. On the other hand, PD-1 antibody blockade improved the survival of mice with EGFR-driven adenocarcinoma by both targeting tumor cells and inducing the activity of T-cells, modulating the expression of immuno-regulatory cytokines. Globally considered, these findings suggest that concurrent inhibition of PD-1 and EGFR pathways may represent a rational and promising approach for EGFR-addicted NSCLC (39).

Our group performed next-generation sequencing to assess the mutational status of a series of EGFR-mutant advanced lung cancers receiving first line gefitinib. The results of our study suggested that the presence of additional coexisting mutations significantly decreases the expected benefit of tyrosine-kinase inhibitors. This finding has a biological rationale. While the presence of a high mutation burden may predict benefit from immunotherapy in unselected lung cancer in the context of an oncogene-

addicted disease, additional coexisting mutations suggest an underlying molecular heterogeneity, leading to by-passing of the main oncogenic stimulus (40).

In contrast to EGFR-activating mutations, KRAS mutations are usually detected in smokers and associated with poor prognosis and no benefit from tyrosine kinase inhibitors and adjuvant chemotherapy (1,41). An integrative analysis of genomic, transcriptomic and proteomic data was recently performed in both chemotherapy-naïve and heavily pre-treated KRAS-mutant lung adenocarcinoma (42). Three biologically distinct subsets of KRAS-mutant cancer were identified by co-occurring genetic alterations in STK11/LKB1 (KL subgroup), TP53 (KP subgroup) and CDKN2A/B inactivation with low TTF1 expression (KC subgroup). Regarding immune system engagement, KP tumors were characterized by an intense inflammatory response with enhanced expression of several costimulatory and coinhibitory factors, including PD-L1. In contrast, KL KRAS-mutant lung adenocarcinoma appeared almost immune-inert. Despite the similar exposure to smoking, KP lung adenocarcinoma showed a higher global mutation rate compared with KL tumors and this finding may contribute to explain the reported differences in terms of immunogenicity between these two subgroups of KRAS-mutant cancer (42).

Only preliminary evidence is available about the immune related aspects of the ALK fusion oncogene that seems to possess an intrinsic immunogenicity value inducing T-cell responses and humoral immunity (43).

Globally considered, the results obtained in the available studies exploring the immunogenicity of oncogene-addicted lung cancer are still preliminary and debatable. A prospective validation in the context of a larger population is mandatory in order to definitively validate the role of major lung cancer oncogenes as reliable parameters to predict the awaited effect derived from PD-1/PD-L1 inhibition.

## Conclusions

Increasing evidence is available to support the role of neoantigens in inducing and maintaining anti-tumor responses to immunotherapeutic agents. In this regard, the burden of random mutations arising during normal DNA replication of non-cancerous stem cells—which lead to the development of cancer—is not perhaps as ‘unlucky’ as it might at first seem, at least as far as the implications for immunotherapy are concerned (44).

If the quantity of neoantigens statistically correlates with the probability of response to immunotherapy, strategies aimed to enhance the production of tumoral neoantigens may theoretically be combined with immunotherapy to improve the expected benefit. In this regard, one of the most promising approaches is radiotherapy. Radiation therapy targeted selectively to the tumor acts as an *in situ* tumor vaccine by inducing release of antigens during cancer cell death in association with pro-inflammatory factors able to trigger the innate immune system to activate tumor-specific T-cells. If successful, not only does it result in the rejection of the irradiated tumor, but also in the rejection of the systemic disease (a phenomenon known as abscopal effect) (45).

Nevertheless, pivotal trials demonstrated that the quality of neoantigens probably matters more than the global mutation burden. Technological advances in genomics and bioinformatics have provided promising tools to efficiently select the strongest immunogenic neoantigens from the broad spectrum of somatic mutations in a tumor. The aim is a ‘reverse immunology’ approach going from theory (computational epitope prediction) to practice (*in vitro* validation).

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## Footnote

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

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