Review Article

Molecular markers and prediction of response to immunotherapy in non-small cell lung cancer, an update

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Abstract: Immunotherapy represents one of the most promising therapeutic approaches in lung cancer, however 50% of lung cancer patients will not respond to this treatment, while others will have transitory or durable responses. Because side effects may be life threatening and treatment costs remain very high, the identification of predictive markers is mandatory and actually extensively studied. Factors that determine response to immune checkpoint inhibitors (ICI) are numerous including tumor microenvironment, immune tumor infiltrates, expression of immune checkpoint proteins (PD-1/PD-L1), gene expression signatures and molecular tumor profiles. Based on high impact factor publications and recent literature this review focuses on the potential predictive value of tumor molecular alterations and tumor mutation burden as predictive markers of response or resistance to ICI. We also discuss the role of circulating tumor DNA (ctDNA) to monitor ICI responses and propose an algorithm that integrates molecular markers upcoming recommendations for first line treatment.

Keywords: Lung cancer; immunotherapy; next generation sequencing (NGS); oncogene drivers; tumor mutation load (TML)

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Introduction

Lung cancer remains the most common cause of cancer death worldwide with more than a million deaths per year. In non-smokers, carcinogenesis is often linked to the presence of somatic molecular alterations in specific oncogenic drivers. The use of selective inhibitors such as anti-EGFR, anti-ALK/ROS1 therapies can lead to tumor shrinkage and prolonged survival. In smokers and in patients without druggable target, immunotherapy is a very promising approach. It was first shown that survival was increased in patients receiving an immune checkpoint inhibitor (ICI) in monotherapy as compared to standard of care in second line treatment (1), in first line for subgroups of patients (2) and more recently clinical benefit was observed in patients receiving combination therapies in first or second lines (3). Indeed, combining ICIs with other anticancer strategies such as targeted therapy, chemotherapy, radiation therapy as well as combinations of different ICIs increases effectiveness. Regardless of treatment regimen, nearly half of lung cancer patients will not respond to ICIs and determinants are still being investigated to better select responders. Sensitivity to ICIs is multifactorial involving tumor genetics background, immune cell infiltrates and the level of immune-modulators such as PD-L1 or PD1 expression. The expression of ICI targets PD1-PD-L1 was investigated and related to increased response to ICIs

although it was shown that patients with PD-L1 positive tumors could be non-responders whereas patients with PD-L1 negative tumors could have clinical benefits. Arguments linked to differences between antibodies used to test PD-L1 expression and to tumor heterogeneity were the most convincing. PD-L1 immunohistochemistry (IHC) despite its drawbacks remains the only validated marker use in clinical practice to select responders for first line pembrolizumab monotherapy (2).

For patients with lung cancer, tumor molecular testing is important, to allow individual treatment decisions and is part of patients' care. Enlarged molecular testing is now feasible in routine thanks to the implementation, in clinical practice, of targeted next generation sequencing (NGS) cancer gene panels to investigate genetic tumor profiles. This easy access to somatic mutations in tumors raised the question of the predictive value of mutation types and mutation load on response to ICIs. The predictive value of oncogene drivers, as *EGFR* or *ALK* fusion and mutations such as *TP53*, *STK11/LKB1*, *KRAS*, was analyzed in ICI series of patients. It was demonstrated that immune cell infiltrates depend upon mutation types driving various response levels to ICI. Recently, tumor mutation load (TML), that is the number of non-synonymous somatic point mutations defined by whole-exome sequencing (WES) was linked to ICIs responses in all tumor types, any treatment lines and any type of treatment (combo or monotherapy) (4). However, exome sequencing is time consuming and not yet cost effective for TML evaluation, which hampers its use in clinical settings. For that reason, large targeted NGS panels have been evaluated as surrogate methods to identify potential drivers and to evaluate TML. The accuracy of targeted panels to evaluate TML is a current subject of research. Performance evaluation of different panels showed correct concordance with WES however the definition of high and low TML groups and the choice of a clinically relevant cut-off remain to be validated. We will discuss these different points in line with data from ICIs clinical trials.

As observed for targeted therapy, patients receiving ICIs will ultimately relapse. Studies are on going to identify molecular markers associated to secondary resistance and circulating tumor DNA (ctDNA) is also being evaluated as part of treatment monitoring.

This review focuses on the predictive value and the implementation of molecular markers in clinical practice to help select and monitor patients receiving immunotherapy.

Genetic determinants of response to ICI

One hypothesis is that tumor immune response is activated by antigenic peptides arising from tumor mutations that act as neoantigens, however not all mutations are efficiently turned into neoantigens. As an example, driver oncogenes often lack immunogenicity (5,6). Interaction between molecular profiles and response to ICIs can be analyzed at gene level. Are specific gene alterations related to response to ICIs? Or at a global level, how is TML linked to response to ICIs?

First trials identified smoking as a predictive marker of response to ICIs (1) and rapidly it was showed that non-smokers with *EGFR* mutated or *ALK* rearranged tumors do not do well with ICIs and should not receive first line ICIs even though tumor cells may express high PD-L1 levels [see Miura *et al.* (7) for review]. Indeed up-regulation of PD-L1 is not rare in *EGFR* mutated or *ALK* rearranged lung tumors and linked to oncogene induced up-regulation, activation of ERK or mTOR signaling (8,9). Most trials have excluded EGFR, ALK and ROS1 positive tumors and literature is scarce on the subject and relies on small subgroups of patients. In second line, the use of ICIs remains controversial. The CheckMate 057 and KEYNOTE-010 trials, which tested the benefit of nivolumab and pembrolizumab over docetaxel chemotherapy in second line did not show any differences between study arms among EGFR-mutant or ALK-fusion patients (1,10).

Patients with *EGFR* mutated tumors and no identified secondary resistance mechanism should be offered chemotherapy rather than an ICIs (11). It seems clear that for patients with oncogene drivers or druggable secondary resistance mechanisms, targeted therapy is the first choice. This lack of efficacy could be due to low levels of CD8+ tumor-infiltrating lymphocytes, and to non-inflamed microenvironment that limits the efficacy of ICIs (12). However recent results from the IMpower150 trial may change treatment option for patients with tyrosine kinase inhibitor (TKI) relapse. Indeed, this trial included *EGFR* and *ALK* positive patients that had progressed after TKI and showed that progression-free survival (PFS) benefit was observed with atezolizumab + chemotherapy + bevacizumab *vs.* chemotherapy + bevacizumab in this subgroup of patients, with a mean PFS of 9.7 versus 6.1 months, HR (95% CI) 0.59 (0.37–0.94) (13). Combo using TKIs and ICIs are on-going to evaluated clinical benefits of the association. *Tables 1-3* summarized the main

Table 1 Summary of the main phase 2/3 clinical trials evaluating pembrolizumab in lung cancer

Study	Reference	Phase	N	Purposes	Results
KEYNOTE-021: NCT02039674	Langer *et al.* 2016	2	123	Carboplatin and pemetrexed with or without pembrolizumab	An objective response was achieved in 55% (33/60) of patients in the pembrolizumab plus chemotherapy group compared with 29% (18/63) in the chemotherapy alone group (P=0.0016)
NCT02879994	Lisberg *et al.* 2018	2	11	Pembrolizumab in EGFR-mutant, PD-L1 positive (>1%), TKI naïve patients	Lack of efficacy of pembrolizumab in TKI naïve, EGFR-mutant, PD-L1 patients even in case of PD-L1 expression >50%
KEYNOTE-010: NCT01905657	Herbst *et al.* 2016	2/3	1,034	Pembrolizumab *vs.* docetaxel for previously treated PD-L1 positive (>1%) NSCLC	OS was significantly longer for pembrolizumab *vs.* docetaxel (HR P=0.0008) No significant difference was achieved in PFS between pembrolizumab and docetaxel groups In the subgroup of patients with PD-L1 positive tumours (expression >50%), OS and PFS were significantly longer with pembrolizumab than with docetaxel (P<0.001)
NCT02142738	Reck *et al.* 2016	3	305	Pembrolizumab *vs.* chemotherapy for PD-L1 positive (>50%) previously untreated advanced NSCLC	Median PFS was 10.3 months with pembrolizumab *vs.* 6 months with chemotherapy (P<0.001) Estimated OS at 6 months was 80.2% in the pembrolizumab group *vs.* 72.4% in the chemotherapy group (P=0.005) The RR was 44.8% with pembrolizumab *vs.* 27.8% with chemotherapy
NCT02578680	Gandhi *et al.* 2018	3	616	Pembrolizumab plus chemotherapy in previously untreated advanced non-squamous NSCLC	Estimated OS rate at 12 months was 69.2% in the pembrolizumab plus chemotherapy group *vs.* 49.4% in the chemotherapy group (P<0.001) An increased expression of PD-L1 significantly improved OS Median PFS was significantly longer in pembrolizumab plus chemotherapy group compared with chemotherapy group (8.8 *vs.* 4.9 months respectively, P<0.001)

OS, overall survival; TKI, tyrosine kinase inhibitor; PFS, progression-free survival; NSCLC, non-small cell lung cancer; RR, response rate.

phase 2/3 clinical trials which evaluated pembrolizumab (*Table 1*) (2,3,14-16), atezolizumab (*Table 2*) (13,17-20) and nivolumab (*Table 3*) (1,10,21-23) in lung cancer.

In smokers, *KRAS* and *TP53* are frequently mutated and their predictive value on response to ICIs was studied in different publications but results are inconsistent. It was suggested that *KRAS-TP53* co-mutation could predict response to immunotherapy. Indeed *KRAS/TP53* mutated samples demonstrated a favorable immune infiltrate and a higher mutation burden (24). However, in another publication *TP53* mutations were modestly associated with increased response and were related to high TML (24) whereas *KRAS* had no predictive value. Finally, *TP53* mutated, *STK11* and *EGFR* wild type tumors were associated with a higher density of CD8 T-cell and PD-L1 expression. This subgroup was associated with a high TML and a longer PFS was reported in immunotherapy treated patients (25). At this point, the predictive value of *KRAS* and *TP53* is not robust enough to be used in clinical practice to select patients for ICIs.

Due to the low frequency of *BRAF*-mutant non-small cell lung cancer (NSCLC), the immune response and immunological characteristics of tumors has not been extensively studied. A recent paper based on a retrospective analysis of 39 patients with BRAF mutated cancer that received ICIs showed that *BRAF* mutant NSCLC is associated with high level of PD-L1 expression and that ICIs have favorable activity with an objective response rate of 25% and 33% in *BRAF* V600E and *BRAF* non-V600E mutant respectively (26).

At the opposite, *LKB1/STK11* mutations in association or not with *KRAS* were related to a lack of response to

Table 2 Summary of the main phase 2/3 clinical trials evaluating atezolizumab in lung cancer

Study	Reference	Phase	N	Purposes	Results
BIRCH: NCT02031458	Peters *et al.* 2017	2	659	Atezolizumab as first line or subsequent therapy for PD-L1 positive (>5%) advanced NSCLC	ORR was 22%, 19% and 18% for first-, second- and third-line therapy respectively. ORR was increased in PD-L1 >50% subgroup Median OS was 23.5, 15.5 and 13.2 months for the three groups respectively
POPLAR: NCT01903993	Fehrenbacher *et al.* 2016	2	144	Atezolizumab *vs.* docetaxel for patients who progressed on post-platinum chemotherapy	OS was 12.6 months for atezolizumab *vs.* 9.7 months for docetaxel (P=0.04) Increasing improvement in OS was associated with increasing PD-L1 expression
OAK: NCT02008227	Rittmeyer *et al.* 2017	3	1,225	Atezolizumab *vs.* docetaxel in patients with previously treated NSCLC	OS was 13.8 months with atezolizumab *vs.* 9.6 months with docetaxel (P=0.0003) OS in the PD-L1 positive tumors (>1%) was 15.7 months with atezolizumab *vs.* 10.3 months with docetaxel (P=0.0102) OS in the PD-L1 low (<1%) or negative tumors was 12.6 months with atezolizumab *vs.* 8.9 months with docetaxel
NCT02366143	Socinski *et al.* 2018	3	1,202	Atezolizumab for first line treatment of metastatic non-squamous NSCLC	The addition of atezolizumab to BCP (bevacizumab + carboplatin + paclitaxel) for first line treatment improved PFS (8.3 *vs.* 6.8 months respectively, P<0.001) and OS (19.2 *vs.* 14.7 months respectively, P=0.02), regardless of PD-L1 expression and EGFR or ALK genetic alteration status
Pooled POPLAR, OAK: NCT01903993, NCT02008227	Gandara *et al.* 2018	3	273, 797	Atezolizumab *vs.* chemotherapy	In patients recruited in OAK with tumors showing a TML >16 mut/Mb (TML plasma test): PFS HR 0.65, 95% CI (0.47–0.92) favors atezolizumab In patients recruited in OAK with tumors showing a TML<16 mut/Mb (TML plasma test): PFS HR 0.98, 95% CI (0.80–1.20)

NSCLC, non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; TML, tumor mutation load; HR, hazard ratio; ORR, objective response rate; mut, mutations.

immunotherapy (27). This could be related to specific immune environment linked to *LKB1/STK11* mutated tumors (28,29). Hellmann *et al.* showed that none of the tumors with STK11/LKB1 alterations in their series responded to treatment (23). They made similar observation with *PTEN* mutations although it did not reach significance. Consistent with these findings another group has compared patients with durable clinical benefit (DCB) versus no durable benefit (NDB) based on targeted NGS data (MSK-IMPACT). Authors showed that *KRAS* had no predictive value but that *STK11* mutations were related to NDB. No other association was mentioned (30).

Altogether tumor cells molecular profiles can significantly impact the tumor immune microenvironment (i.e., T-cells, macrophages and neutrophils density) however large series are needed to validate the predictive value of individual gene mutation as markers of response to ICIs. Among frequently mutated genes, *STK11/LKB1* has the strongest predictive value with consistent observations of a lack of response to ICIs.

Tumor mutational load (TML) as a predictive marker

Determination of TML

Tumor mutation load or tumor mutation burden (TML, TMB), is the number of non-synonymous somatic point mutations based on exome sequencing. It is expressed as a number of mutations per mega base (Mb). When TML is estimated by smaller sequencing panels using targeted NGS, the total number of alterations is reported to panel coverage. Therefore, results can be compared to WES.

Table 3 Phase 2/3 clinical trials evaluating nivolumab in lung cancer

Study	Reference	Phase	N	Purposes	Results
CheckMate 032: NCT01928394	Antonia *et al.* 2016	1/2	216	Nivolumab alone *vs.* nivolumab plus ipilimumab in small-cell lung carcinoma which had progressed after platinum-based chemotherapy	OR was achieved in 10% (10/98) of patients receiving nivolumab alone (3 mg/kg), 23% (14/61) of patients receiving nivolumab (1 mg/kg) and ipilimumab (3 mg/kg) and 19% (10/54) of patients receiving nivolumab (3 mg/kg) and ipilimumab (1 mg/kg)
CheckMate 057: NCT01673867	Borghaei *et al.* 2015	3	582	Nivolumab *vs.* docetaxel in patients with non-squamous NSCLC that had progressed after platinum-based doublet chemotherapy	OS was 12.2 months with nivolumab *vs.* 9.4 months with docetaxel (P=0.002)
					OS rate at 12 months was 51% in the nivolumab group *vs.* 39% in the docetaxel group. OS at 18 months was 39% *vs.* 23% respectively
					RR was 19% with nivolumab *vs.* 12% with docetaxel (P=0.02)
					Median PFS was 2.3 months with nivolumab *vs.* 4.2 months with docetaxel
CheckMate 017: NCT01642004	Brahmer *et al.* 2015	3	272	Nivolumab *vs.* docetaxel in patients with squamous NSCLC that had progressed after first-line chemotherapy	Median OS was 9.2 months with nivolumab *vs.* 6 months with docetaxel
					OS rate at 12 months was 42% with nivolumab *vs.* 24% with docetaxel
					RR was 20% with nivolumab *vs.* 9% with docetaxel (P=0.008)
					Median PFS was 3.5 *vs.* 2.8 months respectively (P<0.001)
					All of these results were independent of PD-L1 expression level
CheckMate 026: NCT02041553	Carbone *et al.* 2017	3	541	First-line nivolumab *vs.* chemotherapy in PD-L1 positive (>1%) NSCLC	Median PFS was 4.2 months with nivolumab *vs.* 5.9 months with chemotherapy (P=0.25) among patients with a PD-L1 tumor-expression >5%
					Median OS was 14.4 *vs.* 13.2 months
					In patients with high TML, PFS was 9.7 *vs.* 5.3 months
					In patients with low-medium TML, PFS was 4.1 *vs.* 6.9 months
CheckMate 227: NCT02477826	Hellmann *et al.* 2018	3	75	Multipart phase 3; different nivolumab based regimen *vs.* chemotherapy; nivolumab-ipilimumab *vs.* chemotherapy in patients with TML high	In all patients PFS was 4.9 months (95% CI, 4.1 to 5.6) with nivolumab plus ipilimumab and 5.5 months (95% CI, 4.6 to 5.6) with chemotherapy
					In patients with tumors showing a TML>10 mut/Mb (FoundationOne): PFS was 7.2 months (95% CI, 5.5 to 13.2) *vs.* 5.5 months (95% CI, 4.4 to 5.8) irrespective of tumor PD-L1 expression level
					In patients with tumors showing TML >13 mut/Mb: no difference in PFS was shown between nivolumab monotherapy *vs.* chemotherapy

NSCLC, non-small cell lung cancer; TML, tumor mutation load; OS, overall survival; PFS, progression-free survival; RR, response rate. OR, odds ratio.

WES sequencing is actually the goal standard; however, it remains expensive and time-consuming, which hampers its clinical practice applications. Different strategies have been tested and compared to WES to validate TML determination by targeted NGS panels. Results showed that methods were concordant when large comprehensive panel of over 1 to 1.5 Mb corresponding to more than 350 genes were used. However smaller panels were reported as surrogate methods, they are used in association with specific bioinformatics pipelines, algorithms to adjust mutation value per gene (31-33). If all studies agree on the positive predictive value of high TML, the validation of a clinically meaningful cut-off and the definition of "high" remains debated and differs between series or clinical trials (30,34). One difficulty is that TML is a continuous variable with an intermediate TML group. Cutoffs were first chosen to optimize specificity and sensitivity retrospectively in clinical trials. However, those used in prospective trials could be different from those used in retrospective trials. As examples, in Rizvi *et al.* (30) the cut-off was set at 178 mutations per exome (approximately 6/Mb), in the CheckMate 026 ancillary study to 243 mutations (7–8/Mb), in Chalmers *et al.* a study demonstrating that TML measurements from comprehensive NGS using the FoundationOne assay (Cambridge, MA, USA) was correlated to WES, TML high was defined as 20 mutations per/Mb (35).

Any TML quantification methods, WES or NGS panels can accurately identify low, intermediate and high TML samples however the definition of a precise threshold may not fit clinical practice. Indeed, mutation count could slightly differ due to the use of different methods, threshold may differ between situations first or second line, combination or monotherapy, differ between tumor types or subtypes and could be heterogeneous between primary and metastasis. The identification of a precise cut-off will be very difficult to validate or will be linked to a companion test which could ultimately limit the access to immunotherapy for patients with lung cancer. At the opposite any laboratory can validate a global TML classification as high/intermediate/low according to internal controls and to its own series of patients allowing large use of this maker to predict response to ICIs. Moreover, TML classification should be integrated to therapeutic options: monotherapy, combination therapy, first or second line and associated with other markers as PD-L1 expression, immune infiltrates to refine treatment selection. Indeed combination of markers may be more accurate as compared to TML only (24).

A few publications evaluated TML on cell free DNA. It was done mainly using comprehensive targeted panels. However due to the frequent low mutation allele frequencies (MAF) in ctDNA, it requires sequencing at high depth, the development of specific analysis pipelines and increases costs. However, for samples known to have high MAF in plasma, TML measurements should be possible using standard pipelines and may be an option. CtDNA is often described as a surrogate marker of tumor heterogeneity and could measure the sum of alterations in different tumor or metastatic sites. The predictive impact of TML measured in circulating DNA remains to be validated however it was recently evaluated as an ancillary study of the OAK trial using targeted NGS and 1.125 Mb of coding region corresponding to 394 genes. The authors showed that 59% of variants were shared between tumor and plasma DNA, that plasma and tissue TML were correlated and that the best cutoff to selected responders to atezolizumab was 16 mutations/Mb (35).

TML and response

In lung cancer, somatic mutation load was related to tobacco exposure and to a specific molecular smoking signature (36). Despite a great variability, lung cancers frequently exhibit high TML related to tobacco exposure. Rizvi *et al.* demonstrated on a small series of patients using WES that TML was predictive for treatment response. TML high tumors are predicted to have more neoantigens expressed. It was shown that neoantigen load correlated with TML, high PD-L1, CTLA4 and CD8+ infiltrates (33).

TML was explored in clinical trials and in different treatment combinations. In first line monotherapy, the ancillary study of CheckMate 026 explored its predictive value in a population of lung cancer patients with a PD-L1 expression of 5% or more. Main result from this phase 3 trial was that nivolumab was not associated with significantly longer PFS as compared to chemotherapy. However, TML was assessed in a sub group of patients using WES. PFS was longer in the subgroup of patients with high TML defined as >243 mutations per exome corresponding to about 8 mutations/Mb (median, 9.7 *vs.* 5.8 months; hazard ratio for disease progression or death, 0.62; 95% CI, 0.38 to 1.00). There was no difference of PFS between the low and medium TML groups. The absence of difference observed for overall survival (OS) was attributed to treatment crossover. No overlap was found between PD-L1 expression and TML however patients with both

PD-L1 >50% and TML high experienced longer PFS (22). A retrospective study evaluating patients recruited in the MKS-IMPACT trial that received ICIs, tested the value of TML to predict clinical benefit defined as complete, partial or stable disease that lasted more than 6 months. In patients with clinical benefit TML was significantly higher, 8.5 *vs.* 6.6/Mb and odd ratios for clinical benefit increased with TML. The rate of clinical benefit and PFS were improved for patients with TML above the 50th percentile and even more for those in the top decile of TML (30). These results are consistent with the hypothesis that a high TML enhance tumor immunogenicity and increase clinical benefit of immunotherapy (36).

In patients receiving combination therapy (nivolumab and ipilimumab) in the CheckMate 012 trial, TML determined by WES was the strongest marker of response using the median (158 mutations/exome) as cut-off. However a continuous effect of TML on PFS was demonstrated (24). The impact of TML was also described with atezolizumab, a cut-off of 16/Mb discriminates patients with favorable outcome with atezolizumab as compared to chemotherapy (37). Similar observations were made in studies combining ICIs to chemotherapy. Subgroup analyses from CheckMate 227 reported that patients with high TML (>10 mutations per Mb) demonstrated clinical benefits with the nivolumab + ipilimumab or the nivolumab + chemotherapy associations versus chemotherapy alone. No difference was observed in the group of patients with low TML (38). *Tables 1-3* reports main trials in NSCLC and potential associated biomarkers.

Based on different studies, high TML predicts response to ICIs used in monotherapy or in combinations, however some patients with low TML respond to treatment and some with high TML have short PFS.

TML is the surrogate marker of tumor neoantigen load (TNagL). Different algorithms taking into account various parameters including peptide binding to patients' specific HLA isoforms have been developed to estimate neoantigen load. It was shown that TNagL is much lower than TML with only a few neoantigens present even when TML is high (39). High TML increases the chance that, at random, neoantigens are synthetized by tumor cells. Due to the importance of neoantigens in activating immune responses, TNagL is an attractive biomarker to better identify responders to ICIs.

Repair pathway defects and TML

Repair deficiencies are often associated to cancer and linked to high TML. It is especially true for mismatch repair (MMR) and replicative polymerases (POLE, POLD1) deficiencies. MMR deficiency is associated to lynch syndrome and occurs in sporadic cancers mainly colorectal, endometrial, ovarian and gastric tumors. MMR deficiency causes replication errors to accumulate, is linked to a microsatellite instable (MSI) phenotype and to high TML. The MSI phenotype is a predictive marker of response to ICIs in various cancer types and can easily be assessed by standard routine methods (40). Data from the AACR GENIE database shows that 2.1%, 1.09%, 1.65% and 1.87% of lung tumors have mutations in the MMR genes *MSH2*, *MLH1*, *PMS2* and *MSH6* respectively (41). However, the identification of an MSI phenotype is rare in lung cancer (<0.5%) and is often associated to other predictors (TML and PD-L1) so the identification of the MMR status in lung is not justified (42,43). Mutations in replicative polymerases have been described in patients treated by ICIs. In the Rizvi series, patients with POLE and POLD1 mutations had long term responses. The identification of POLE and POLD1 exonuclease domain mutation is accessible using targeted NGS in routine diagnostics. To conclude, the identification of repair pathway defects such as MMR deficiency which is rare considering lung cancer and mutation in DNA polymerases POLE and POLD1 are surrogate markers of TML (30).

Resistance to immunotherapy

Three different resistance mechanisms to immunotherapy were described: (I) the primary resistance is defined as an absence of response to immunotherapy; (II) the adaptive immune resistance mechanism, in which cancer cells are recognized by the immune system but escape to immune attack; and (III) the acquired resistance, in which a tumor initially responds to immunotherapy but relapses after a period of time. These mechanisms may be related to tumor-cell intrinsic or extrinsic factors such as the absence of antigenic proteins, the absence of antigen presentation, a genetic T-cell exclusion or an insensibility to T-cells… (44). Some of these factors could be sustained by different molecular alterations.

Molecular features of resistance to immunotherapy

Activation of Wnt/β-catenin pathway

Several oncogenic signaling pathways have been associated with resistance mechanisms to immunotherapy. For

instance, an activation of the Wnt/β-catenin pathway was described as a mechanism of primary or acquired resistance to immunotherapy. Activation of this pathway through stabilization of β-catenin modulates formation of transcriptional complexes, leading to suppression of CCL4 transcription and secretion. CCL4 is a chemokine implicated in the recruitment of immune cells like NK cells and monocytes. CCL4 expression is associated with improved response to immunotherapy. The lack of chemokine secretion decreases immune cells recruitment, activation and may be responsible for immunotherapy resistance. In murine melanoma models, the lack of Wnt/β-catenin signaling is associated with a good response to immunotherapy whereas an activation results in resistance (45). Molecular alterations such as *CTNNB1* mutations especially in exon 3 or copy number variation (i.e., deletions) are known to promote β-catenin stabilization and are found in lung cancer. In the same way, adenomatous polyposis coli (APC) mutations also decrease ubiquitin-proteasome degradation of β-catenin (46).

Constitutive expression of PD-L1 through Akt/mTOR pathway activation

The constitutive expression of PD-L1 by cancer cells leads to an active inhibition of immunotherapy response. In this context, different molecular alterations have been pointed out to trigger PD-L1 constitutive expression. For example, activation of the Akt-mTOR signaling pathway regulates PD-L1 expression *in vitro* and *in vivo* (47) and promotes immunotherapy resistance. Loss of PTEN through deletion or inactivating mutations is commonly found in different cancers including lung and is associated with an increased PD-L1 expression (48). In the same pathway, activating mutations of *PIK3CA* or *AKT* could also enhance Akt/mTOR signaling and tightly regulate PD-L1 expression in lung cancer (47). Moreover, PI3K-AKT pathway inhibitors were shown to improve the efficacy of immunotherapy in murine models (49).

Impairment of the interferon-gamma (IFN-γ) signaling pathway

The IFN-γ signaling pathway plays a key role of the immune response. IFN-γ is produced and released by T-cells and induces anti-tumor immune response. The binding of IFN-γ on cell surface IFN-γ receptors (IFNGR1 and 2) induces receptor dimerization allowing activation of the interferon receptor-associated JAK (Janus kinase 1 or 2). JAKs then activate STATs (signal transducer and activator of transcription proteins) which translocate to the nucleus to induce transcription of IFN-γ targeted genes (50). Both primary and acquired resistances to immunotherapy may be mediated through JAK/STAT pathway impairment (51). For instance, inactivating mutations of JAK1 p.Gln503* or JAK2 p.Phe547_splice c.1641+2T>G result in a lack of response to IFN-γ stimulation in melanoma (52). Inactivating mutations in genes of the IFN-γ signaling pathway protect tumor cells from IFN-γ mediated anti-tumor immunity (53).

Loss of MHC class I expression in tumor cells

Beta-2-microglobulin (β2m) is a component of MHC class I complex widely implicated in processing and antigen presentation to the cell surface. Downregulation of β2m through acquired mutation of the *B2M* gene leads to a lack of MHC class I expression in tumor cells and also provide resistance to immunotherapy in lung cancer mouse model (54).

CtDNA concentration as a predictive marker of immunotherapy response

CtDNA is now commonly used to manage TKI treatment in *EGFR* mutated NSCLC. In a cohort of nivolumab treated NSCLC patients, low ctDNA concentration at first evaluation (<2 ng/mL) is associated with clinical benefit and increased overall response rate. Moreover, a decrease of ctDNA concentration between diagnosis and first evaluation is also correlated with PFS, clinical benefit and tumor response (55). CtDNA concentration may also be used in association with medical imaging for the assessment of lung cancer immunotherapy response. A >50% decrease in mutant allele fraction from baseline seems to be correlated with radiological response to immunotherapy (56). Quantification of ctDNA in patients receiving ICIs is feasible in clinics thanks to the development of targeted NGS panel in routine diagnostics.

Discussion/conclusions

ICIs are widely used and evaluated in lung cancer. Immunotherapy is a therapeutic option for patients with metastatic lung cancer, in monotherapy or in combinations and is also extensively evaluated in patients with localized disease. However not all patients will benefit from these treatments, toxicities may be life threatening and treatment costs are important. It is therefore mandatory to refine patients' selection and to personalize the use of ICIs.

PD-L1 expression level is currently the only validated

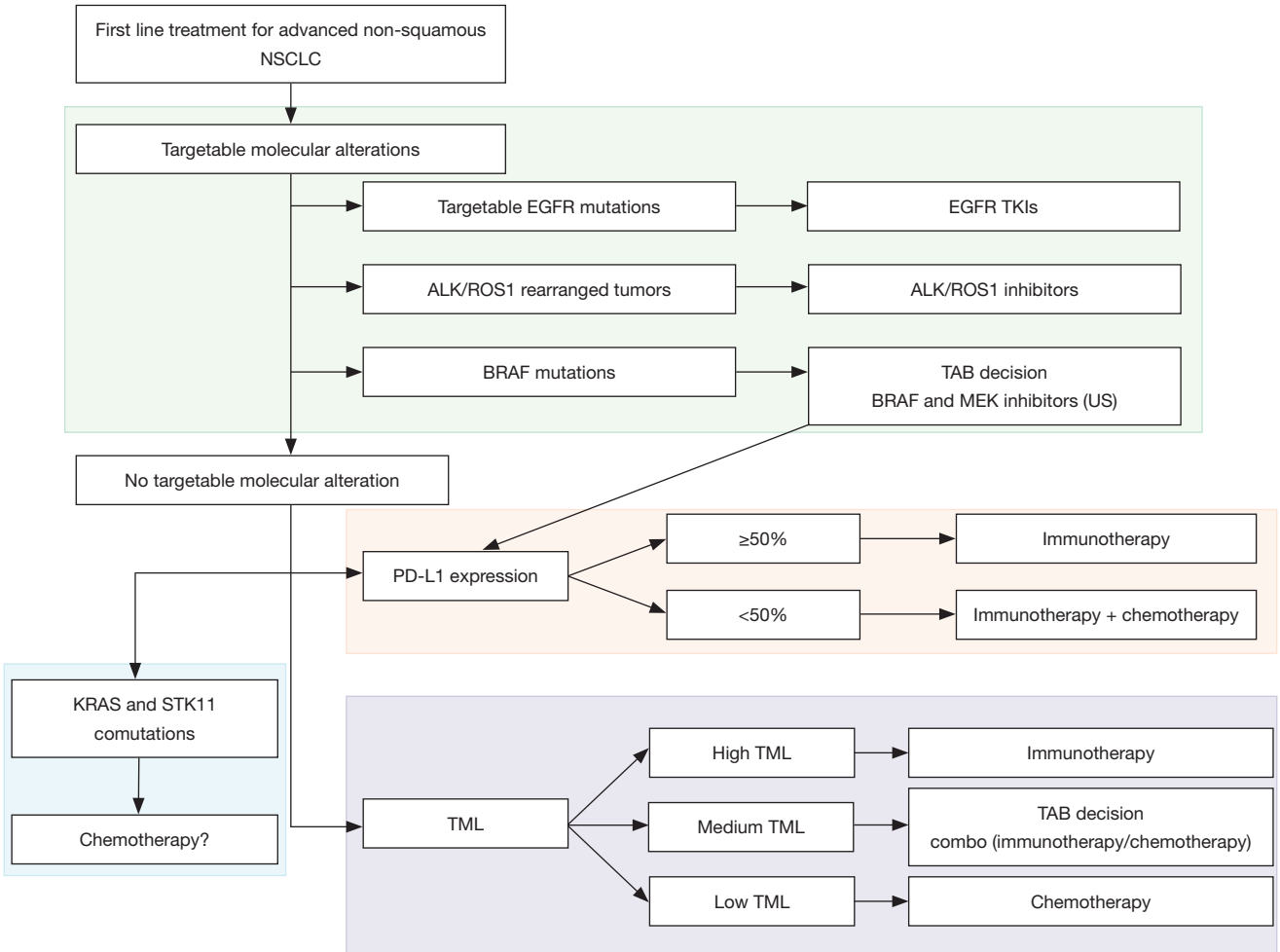

Figure 1 First-line treatment algorithm of advanced non-squamous NSCLC. NSCLC, non-small cell lung cancer; TML, tumor mutation load; TKI, tyrosine kinase inhibitor.

marker in first line for NSCLC. Immunotherapy is recommended in first line for patients with an *EGFR* or *ALK* wild type, PD-L1 >50% tumor. There is no validated recommendation for combination therapies or second line treatments although trials try to point out groups of patients with a higher clinical benefit. In this context, will molecular testing, mutation profiling and TML evaluation help refine patient selection? Based on the recent consensus statement on immunotherapy for the treatment of NSCLC published by the society for immunotherapy of cancer (57), *Figure 1* summarized first-line treatment options and associated potential markers. Regardless of PD-L1 expression patients with targetable alterations are to receive targeted therapy as first line treatment. Last year, the FDA approved the association of dabrafenib and trametinib in first line treatment of *BRAF* mutated advanced NSCLC. However, for these patients, immunotherapy could be also considered as an option in tumors that expressed high levels of PD-L1. To our knowledge, no study compared immunotherapy *vs.* BRAF and MEK targeted therapy in patients with *BRAF* mutated tumors. A case-by-case TAB decision could be suitable in these cases. Activating *KRAS* mutations are the most commonly found oncogenic driver in tobacco induced NSCLC. Although studies are inconsistent concerning the predictive value of *KRAS* mutations, different studies underlined the value of *STK11* mutations with or without KRAS alterations. Indeed, patients showed decreased ORR, shorter PFS, shorter OS and poor clinical outcomes regardless of PD-L1 or *KRAS* status or compared to KRAS-only (27). Therefore, chemotherapy may be considered as first line treatment for patients with *STK11* mutated tumors.

TML and PD-L1 expression are two independent

predictors of response to immunotherapy. Their combined use will rely on the development of TML testing. Both may be important to support the decision of immunotherapy or combine therapies in patients without druggable drivers. One challenge will be to select the best treatment for patients with TML low/PD-L1 high or TML high/PD-L1 low cancers for which combination strategies might be more efficient than monotherapies.

The recent development of immunotherapy has greatly improved the management of patients diagnosed with lung cancer. Optimizing treatment and combination therapies remains a real challenge to detect primary or acquired resistance and to monitor treatment during follow-up. There are data supporting the role of molecular testing and mainly TML as a meaningful biomarker of response to immunotherapy. However, response to ICIs depends upon numerous parameters including tumor cells and immune infiltrates and it is likely that patient selection will not rely on a unique biomarker. It is time to develop standardized testing algorithms allowing comparison between drugs and efforts should be done to identify new biomarkers especially in long responder patients. Molecular screening and TML evaluation will be useful to detect molecular alterations involved in ICIs responses. However, to reach clinical value it may first be important to define the population of patients to be selected. Are we expecting tumor stabilization, transitory responses or long-time responses? Biomarkers will probably be different. To optimize immunotherapy and truly show impact on survival we have to learn how to combine biomarkers and identify the best combination. In daily practice, *EGFR*, *ALK*, *ROS1* and *PD-L1* can help, ICIs should not be proposed to patients with *STK11* mutated tumors at least in first line as there are consistent results showing no response in this situation and TML needs to be implemented in clinics but interpretation guidelines are still to be validated. Real progress has been made in the management of lung cancer patients thanks to the development of ICIs, we have to progress in monitoring their use to maximize responses, to minimize side effects and rationalize costs.

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Footnote

Conflicts of Interest: H Blons declares occasional lectures and boards for Roche, AstraZeneca, Boehringer Ingelheim, Pfizer, BMS, and MSD. Pierre Laurent-Puig declares participation lectures and boards for Roche, AstraZeneca, Boehringer Ingelheim, Pfizer, BMS, MSD, MDS Serrono, Amgen, and Biocartis. The other authors have no conflicts of interest to declare.

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