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Drug resistance in non-small cell lung Cancer (NSCLC): Impact of genetic and non-genetic alterations on therapeutic regimen and responsiveness

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ABSTRACT

The discovery of genetic alterations, that can be targeted therapeutically, has launched a new era for lung cancer research and personalized therapy. However, not all the identified new genetic driver mutations are therapeutically targetable due to high toxicity profile. On the other hand, those genetic alterations that could be pharmacologically targeted, are often subject of alternative mutations that lead to drug resistance, which represents one of the major clinical limitation. Mechanisms of acquired resistance in oncogene-driven malignancies occur after additional genetic alterations of the primary oncogene. In this scenario, the secondary genetic alteration can lead to up-regulation of bypass-signaling pathways, changes in tumor histology or alterations in drug metabolism, that are able to promote drug resistance with an ensuing lower survival rate of the patient.
Another aspect to be considered is that non-genetically mutated patients still have poor pharmacological options and therefore still represent an unmet medical need. Therefore, identifying mechanisms underlying both drug resistance in genetically mutated patients and novel therapeutic alternatives for non-mutated NSCLC patients is still an area of intense investigation.

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Abbreviations: AhR, Aryl hydrocarbon receptor; AICDA, Activation-induced cytosine deaminase; AKT1, V-Akt Murine Thymoma Viral Oncogene Homolog 1; ALK, Aanaplastic lymphoma kinase; BRAF, v-Raf murine sarcoma viral oncogene homolog B; cfDNA, Cell-free DNA; CPIs, Checkpoint inhibitors; CTC, Circulating tumor cells; ctDNA, Circulating tumor DNA; CTLA4, Cytotoxic T-lymphocyte antigen 4; EGFR, Eepidermal growth factor receptor; EMA, European Medicines Agency; FDA, Food and Drug Administration; FFPE, Formalin-fixed paraffin-embedded; FGFR1, Fibroblast Growth Factor Receptor 1; HC, Hybrid capture; HER2, Epidermal Growth Factor) Receptor 2; KRAS, Kirsten Rat Sarcoma; LCMC, Lung Cancer Mutation Consortium; MAP2K1, Mitogen-Activated Protein Kinase Kinase 1; MET, Mesenchymal to Epithelial Transition; mTOR, Mammalian target of rapamycin; NCCN, National Comprehensive Cancer Network; NGS, Next generation sequencing; NRF2, Nuclear factor erythroid 2-like 2 (NFE2L2) related factor 2; NSCLC, Non-Small Cell Lung Cancer; ORR, Overall response rate; OS, Overall survival; PD-1, Programmed Death-1; PD-L1, Programmed Death-Ligand 1; PFS, Progression-free survival; PI3K, Phosphoinositide 3-Kinase; PIK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; PTK, Protein tyrosine kinase; RAF1, V-Raf-1 Murine Leukemia Viral Oncogene Homolog 1; RAS, Rat Sarcoma Viral Oncogene Homolog; RET, Rearranged during Transfection; Ros1, V-Ros Avian UR2 Sarcoma Virus Oncogene Homolog 1; TILs, Tumor-infiltrating lymphocytes; TKIs, Tyrosine kinase inhibitors; TMB, Tumor Mutational Burden; TPS, Tumor proportion score; WHO, Word Health Organization; ASCO, American society Clinical Oncology; ESMO, European Society Medical Oncology.

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1. Introduction

Non-Small Cell Lung Cancer (NSCLC) is a devastating disease, which causes 1.59 million cancer-related death globally per year. The Word Health Organization (WHO) estimated 2.093.876 (11.6%) new cases in 2018, with a number of deaths of 1.761.007 (18.4%) (http://gco.iarc.fr) and a five-year survival rate (17.8%) much lower than other leading cancers (i.e. breast, colon). This dramatic condition is primarily due to the lack of early detection tools and to the recognition of the symptoms at the sole late stages. <15% of patients are diagnosed of early stage I of lung cancer, and <15% of all patients survive for 5 years after the diagnosis. To date, >80% of patients are ineligible for surgical resection at the time of diagnosis, mostly because of the advanced stage of the cancer and of the poor general conditions at stage III-IV (Hirsch, Franklin, Gazdar, & Bunn Jr., 2001; Rizvi & Peters, 2017). Several screenings have been developed so far to identify biomarkers to provide insights on diagnosis, prognosis and response to treatment (Tang et al., 2017). In particular, in addition to those for early diagnosis, several screenings are nowadays fundamental to define personalized pharmacological options.

Lung cancer patients are firstly classified according to the resectable or non-resectable tumor mass, which defines them as early or advanced stage lung cancer patients (Fig. 1). One of the main difference between the two groups, that can highly impact on the therapeutic options, is that the biological sample obtained from patients undergoing surgical procedures can be appropriately screened for specific protein/biomarker expression and any type of gene mutation/alteration. In contrast, advanced lung cancer patients, who cannot undergo surgical resection, have limited options in that the small tissue sample obtained from biopsy does not always allow to perform biological considerations on which a therapeutic treatment is chosen, limiting oncologists' pharmacological options(Planchard et al., 2018).

According to WHO classification, once the morphological diagnosis has been performed, a second step requires the identification of specific predictive biomarkers that can first of all direct oncologists towards the right pharmacological option/s and then allow to design a therapeutic protocol for the patient according to the registered response rate to the chosen therapy (Fig. 1) (Planchard et al., 2018). Based on this, actual clinical practice is characterized by two testing streams: the first aims to define any oncogene alteration and the second to evaluate immune-checkpoint/s (i.e. programmed cell death ligand 1, PD-L1) expression. Therefore, one of the main limitation for advanced non-resectable lung cancer is to provide enough tumor sample to perform the above screening (Fig. 1). To circumvent this, many genetic screenings on liquid biopsy have been so far suggested, such as cell free DNA (cfDNA) and circulating tumor DNA (ctDNA), which could verify oncogenic drivers. Because most of the genetic mutations/alterations are not expressed on the hematopoietic cell line, these two examples of novel screenings appear appropriate as they are based on circulating tumor-derived DNA or circulating tumor cells (CTC), but many technical pitfalls limit their routinely usage (Heitzer, Haque, Roberts, & Speicher, 2019). In addition, genetic evaluation of altered expression of immune checkpoint/s is not as reliable as tumor expression of the protein levels. In this regard, both the expression of specific immune checkpoint proteins (i.e. PD-L1 and cytotoxic T-lymphocyte antigen 4, CTLA4) and the infiltration of $CD3^+$ and $CD8^+$ T cells, identified as Immunoscore according to the tumor and its invasive margin analyzed by means of an image-analysis software, are eligible to better define the therapeutic options which are designed to fight tumor immune escape (Galon et al., 2014).

1.1. Genetic mutations

NSCLC patients harbor multiple mutations (Table 1) in 1. epidermal growth factor receptor (EGFR), or 2. anaplastic lymphoma kinase (ALK)/V-Ros Avian UR2 Sarcoma Virus Oncogene Homolog 1 (Ros1) fusion, but even genomic alterations in 3. Kirsten Rat Sarcoma (KRAS), 4. v-Raf murine sarcoma viral oncogene homolog B (BRAF), 5. Human EGF (Epidermal Growth Factor) Receptor 2 (HER2), 6. Rearranged during Transfection (RET) and 7. Mesenchymal to Epithelial Transition (MET) (Pikor, Ramnarine, Lam, & Lam, 2013).

1.2. EGFR mutations

EGFR is over-expressed in 10–30% of NSCLC patients, according to their geographic origin. The incidence of EGFR mutations is predominant in Asian population (40–55%), never smokers (42%), females (22%) and adenocarcinomas (15–20%), squamous carcinoma (0–15%) (Table 1) (Laenger F et al., 2015). Double mutations of EGFR and KRAS are observed in 1.6% of all NSCLC patients (Li et al., 2014). Until ten years ago, EGFR over-expression was considered a promising translational therapeutic target. However, it was subsequently discovered that activating mutations, rather than over-expression of EGFR, were the prime therapeutic targets. Activating mutations in exons 18–21 of the tyrosine-kinase domain of EGFR (Fig. 2) are predictors of the

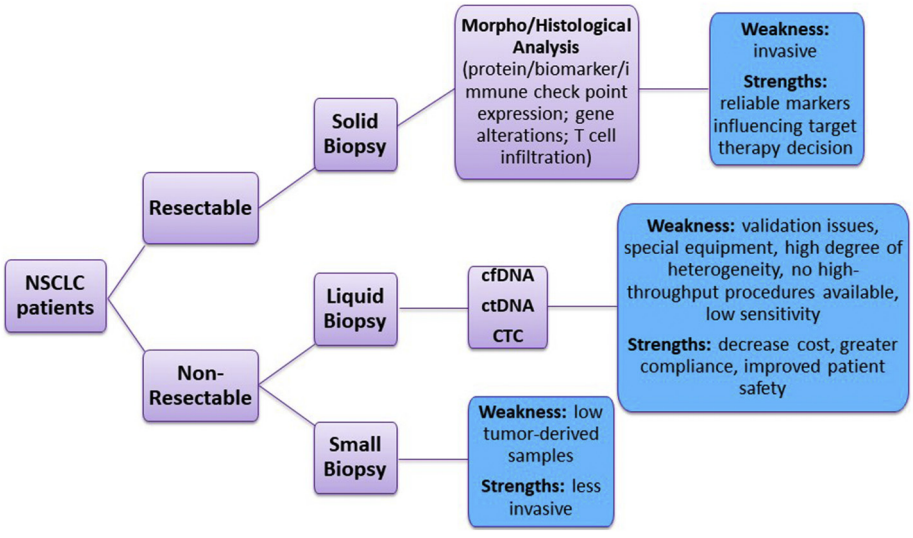

Fig. 1. NSCLC patients are classified as resectable or non-resectable according to the stage and overall life conditions, defining them as early or advanced stage patients. This classification is very important in that the ensuing genetic and molecular alterations are evaluated on solid or liquid biopsy. Weaknesses and Strengths based on the availability of human samples are described.

Table 1
Alterated genes in NSCLC and acquired mutations conferring resistance.

AKT1	E17K mutation	0.6%		N/A	47
ALK	EML4-ALK, KIF5B-ALK, TFG-ALK fusions	3–7%	L1196 M, G1202R, S1206Y, G1269A, L1152R, C1156Y, I1151Tins, L1198F, G1123S, G1123SD, F1174C/V, T1151K, I1171T/N/S, V1180 L	Light smokers (< 10 pack years) and/or never-smokers	47–48–49-50-51
BRAF	V600E, G469A, D594G	1.5–3.5%		Former/current smokers	47–52
DDR2	L63 V, I120M, D125Y, L239R, G253C, G505S, C580Y, I638F, T765P, G774E, G774 V	3.8%		No	47–53
EGFR	G719X (exon 18), in-frame deletions (exon 19), L861Q and L858R single-point mutations (exon 21)	10–35%	T790 M, D761Y, T854A, L747S, C797S, G796D	Female never smokers (defined as <100 cigarettes in a patient's lifetime) with adenocarcinoma histology, former and current smokers	47–50–51-54
FGFR	Amplification (FGFR1); W290C, S320C, K660E, K660 N (FGFR2)	20%		Former/current smokers	47–55
HER2	Exon 20 in-frame insertion	2–4%		Never smokers (defined as <100 cigarettes in a patient's lifetime) with adenocarcinoma histology, former and current smokers	47–56
KRAS	G12C, G12D or G13D, G12 V	15–25%		Former/current smokers and never smokers	47–57
MEK1	K57 N, Q56P	1%		Former/current smokers	47–58
MET	Amplification; R988C, R988C + T1010I, S1058P; KIF5B–MET fusion	2–4%		Male smokers	47–59
NRAS	Q61H/K/L/R (exon 3) and G12A/C/D/R/S (exon 2)	1%		Former/current smokers	47–60
NRF2	D29H, G31A, R34G, E79K, G81S, G241A	2,3%		Smokers with squamous histology	61
PIK3CA	E545K or E542K (exon 9), H1047R or H1047L (exon 20)	1–3%		Never, former and current smokers	47–62
PTEN	K125 N, Y155H	4–8%		Ever smokers	47–63
RET	CCDC6-RET, KIF5B-RET and TRIM33-RET fusion	1%		Never smokers	47–64
ROS1	SLC34A2-ROS1, CD74-ROS1, EZR-ROS1, TPM3-ROS1, and SDC4-ROS1	1%	G2032R, D2033N	Light smokers (<10 pack years) and/or never-smokers.	47–50-65

pharmacological response to anti-EGFR therapy. Physiologically, EGFR activation leads to homo- or hetero-dimerization which ends in cytoplasmic signaling of the PI3K/AKT1/mTOR (Phosphoinositide 3-Kinase/V-Akt Murine Thymoma Viral Oncogene Homolog 1/Mammalian target of rapamycin) or RAS/RAF1/MAP2K1 (Rat Sarcoma Viral Oncogene Homolog/V-Raf-1 Murine Leukemia Viral Oncogene

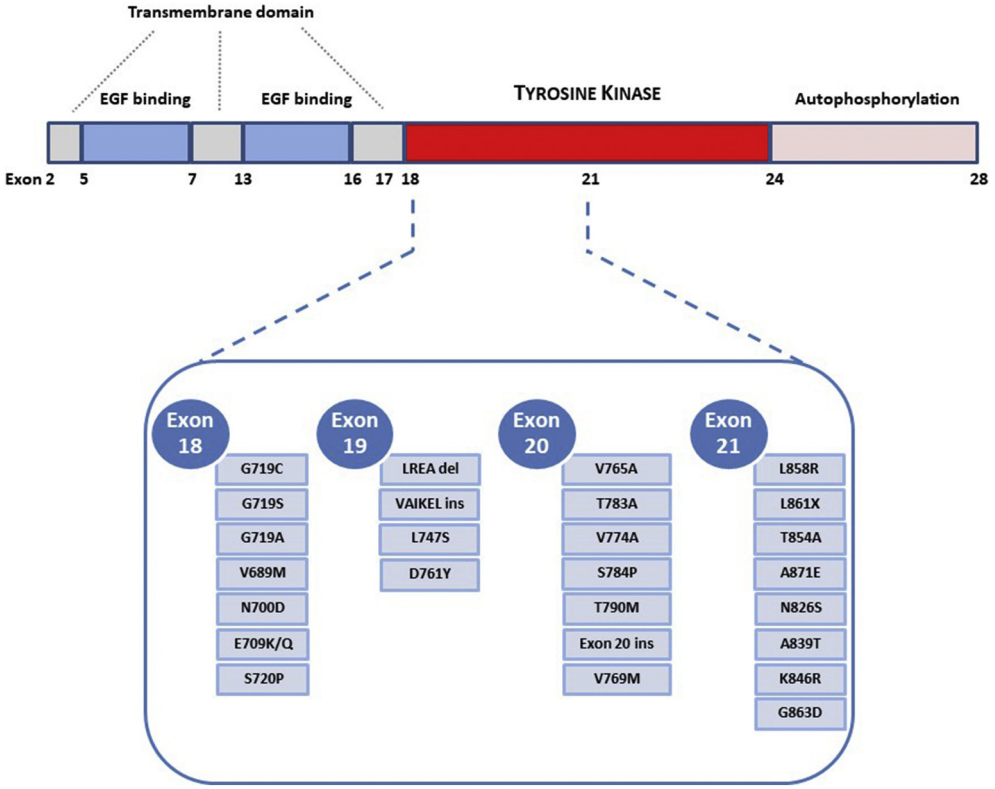

Fig. 2. Activating mutations in exons 18–21 of the tyrosine-kinase domain of EGFR.

Homolog 1/Mitogen-Activated Protein Kinase Kinase 1) pathways which are involved in cell proliferation, angiogenesis and inhibition of apoptosis (Laenger F et al., 2015; Schrank, Chhabra, Lin, Iderzorig, & Osude, 2018). The most common in-frame deletion occurs in exon 19 (delE746-A750) together with point mutations of exon 21 (L858R) (Okabe et al., 2007). Mutations on exon 20 occur after treatment with tyrosine kinase inhibitors (TKIs) and is considered an acquired drug resistance as stated below (see paragraph 3). So far, two basic mechanisms of drug resistance due to EGFR mutations are defined: 1. genetic alteration of the target molecule by point mutations or amplification, or 2. activation of bypass signaling. In the first case, the most important mutation relates to the point mutation T790 M on exon 20, which dramatically reduces the potency/efficacy of TKIs (Pao et al., 2005). In the second case, bypass signaling includes MET amplification (5–22%), PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) mutation (3%), BRAF mutation (1%), over-expression of AXL (AXL Receptor Tyrosine Kinase), encoding a tyrosine kinase receptor, and of HER2 (13%) (Laenger F et al., 2015).

1.3. ALK translocations

ALK is a transmembrane tyrosine kinase receptor, which following ligand binding and homodimerization, induces the activation of the PI3K/AKT1/mTOR or RAS/RAF1/MAP2K1 pathways. Alterations of the ALK gene are described by an inversion within the short arm of the chromosome 2, which encodes ALK, involving EML4, or a translocation and fusion with other genetic partners, thus generating a chimeric protein constitutively activated (Soda et al., 2007). EML4-ALK fused oncogene is present in up to 3–7% of NSCLC patients (Table 1) (Chan & Hughes, 2015). The incidence of this type of genetic alteration appears to be higher in females than males (7.6% vs 4.8%) and in younger (median 55 years) and light or never smokers (8.6% vs 3.6% in smokers) (Laenger F et al., 2015).

1.4. BRAF mutations

BRAF is downstream KRAS and regulates cell proliferation and survival. BRAF mutations are almost frequent in 2–10% of NSCLC patients (Table 1). The mutation V600E is the most common (50%), followed by G469A mutation (39%) and D594G (11%) (Paik et al., 2011). Recently, co-occurring mutations of EGFR, PIK3CA, KRAS and ALK have been registered, implying that BRAF is not a predominant oncogenic driver. BRAF mutations usually occur in adenocarcinomas in Caucasian patients who are former or current smokers (Villaruz et al., 2015).

1.5. ROS1 mutations

ROS1 is a tyrosine kinase receptor sharing 80% of homology to ALK. These mutations are mutually exclusive with KRAS, EGFR and ALK mutations, and occur in light or never smokers (Go et al., 2013).

1.6. PIK3CA mutations

PI3K is an intracellular kinase involved in cell proliferation. Mutations of PIK3CA cause aberrant signaling of PI3K/AKT1/mTOR pathway and in 46–70% of cases they co-exist with other driver mutations, such as EGFR, KRAS and ALK. PIK3CA mutations occur in 3–16% of NSCLC patients with a predominance in squamous carcinoma (Wang et al., 2014).

1.7. HER2 mutations

These mutations occur in 1–4% of NSCLC patients, preferentially in adenocarcinomas, Asians, females, never-smokers (Shigematsu et al., 2005).

1.8. MET mutations

MET amplification occurs in 2–21% of all NSCLCs. It has been associated to poor prognosis and to mechanism of acquired EGFR resistance. There is a preferential occurrence in males, smokers, squamous carcinoma and Asians (12% vs Caucasian 5%) (Krishnaswamy et al., 2009).

Other genetic mutations have so far been registered (Table 1), as in the case of Fibroblast Growth Factor Receptor 1 (FGFR1), Nuclear factor erythroid 2-like 2 (NFE2L2) related factor 2 (NRF2) and AKT1 alterations, typical of squamous cell carcinoma patients (Pikor et al., 2013). While for the majority of the oncogenic driver mutations an effective targeting remain a therapeutic challenge, EGFR- and/or ALK-mutated NSCLC patients are particularly sensitive to treatment with tyrosine kinase inhibitors (TKIs), a relatively new class of small molecules developed to compete with ATP for the ATP binding site of the protein tyrosine kinase (PTK) motif, reducing tyrosine kinase phosphorylation, thereby blocking cell signaling pathways that drive tumor cell proliferation. Several TKIs have been approved or are in clinical trials and have made great advances in the treatment of lung cancer. Unfortunately, despite the initial sensitivity, long-term effectiveness of such therapies is universally limited by the development of resistance that usually develops after more or less one year after the initial treatment, mostly due to additional genetic alterations in the primary oncogene (Table 1), which facilitate continued downstream signaling or induce up-regulation of bypass-signaling pathways with ensuing changes in tumor histology or alterations in drug metabolism (Gainor & Shaw, 2013). Although the molecular mechanisms of resistance are not fully elucidated, recent studies are proposing new mediators associated to TKIs resistance and new generation of TKIs are on their way to evade the resistance and enhance the therapeutic efficiency.

2. Targeted therapy in NSCLC

The new paradigm for NSCLC patients focuses on personalized treatment according to the tumor molecular biology in that clinical options are based on specific driver genomic alteration/s or proteins (i.e. PD-L1/PD-1). Based on the clinical American society Clinical Oncology (ASCO) and European Society Medical Oncology (ESMO) guidelines, patients undergo pharmacological treatment first of all according to the tumor genotyping (mutated vs non-mutated) and then to the expression of specific immune checkpoints (i.e. PD-L1) (Planchard et al., 2018). In particular, ASCO guidelines (https://www.asco.org/practice-guidelines/quality-guidelines/guidelines/thoracic-cancer#/9776) strongly recommend against evaluating EGFR, as well as standalone ROS1 or BRAF testing in advanced lung adenocarcinoma, and RET, ERBB2 (HER2), KRAS, and MET testing as part of larger panels. Recommendations are also provided for testing methods for lung cancers that have a nonadenocarcinoma non–small-cell component, for patients with targetable mutations who have relapsed on targeted therapy, and for testing the presence of circulating cell-free DNA. Lung Cancer Mutation Consortium (LCMC) collectively termed these molecular abnormalities oncogenic drivers to include multiple types of genomic changes critical to cancer development and maintenance, further defining these drivers as actionable, based on the demonstration that the downstream effects of these abnormalities initiate or maintain the neoplastic process, implying a pharmacological benefit by administering agents against each genomic alteration (Kris et al., 2014). Therefore, testing for somatic mutations in EGFR gene and rearrangements of ALK gene is now of routine. As shown in Fig. 3, mutated patients are treated with TKIs, distinguished in TKIs that target EGFR, ALK, ROS1 or BRAF mutation. First-line treatment for these patients foresees:

1. EGFR-tyrosine kinase inhibitors (EGFR-TKIs), that can block EGFR signaling pathway that educates neoplastic cells towards growth. Among these, first generation Erlotinib (Tarceva®) and gefitinib (Iressa®), second generation Afatinib (Gilotrif®) and Dacomitiniv

(Vizimpro®), third generation Osimertinib (Tagrisso®) have been approved and used alone (without chemotherapy) as first-line treatment for advanced NSCLC patients who encounter mutation/s of the EGFR gene. Erlotinib can also be used for advanced NSCLC in the absence of these mutations whether chemotherapy is not effective. Osimertinib is an EGFR inhibitor that works against cells with the most common EGFR alteration, T790 M mutation, that develops after TKIs treatment, and therefore it is used as second-line treatment, besides that some oncologists take advantage that the acquired mutation/resistance takes place after TKI first-treatment, gaining time on long-term therapy. Necitumumab (Portrazza®) is a second generation humanized monoclonal antibody that blocks EGFR signaling. It can be used along with chemotherapy as first-line treatment in patients with advanced squamous lung cancer (https://www.cancer.org/cancer/non-small-cell-lung-cancer/treating/targeted-therapies.html).

2. ALK inhibitors are TKIs able to target ALK gene alterations. First generation drugs is represented by crizotinib (Xalkori), second generation by Ceritinib (Zykadia®), Alectinib (Alecensa®) and Brigatinib (Alunbrig®) and third generation by Lorlatinib (Lorbrena®). To date, Crizotinib is also used for patients who present ROS1 alterations (https://www.cancer.org/cancer/non-small-cell-lung-cancer/treating/targeted-therapies.html).
3. BRAF/MET inhibitors directly inhibit the mutated protein/s. Dabrafenib (Tafinlar®) is a BRAF inhibitor. Trametinib (Mekinist®) is a MEK inhibitor. These drugs can be used together to treat metastatic NSCLC (https://www.cancer.org/cancer/non-small-cell-lung-cancer/treating/targeted-therapies.html).

In this scenario, it has to be noted that, although the introduction of targeted treatment due to the identification of driver oncogenes, the percentage of mutated NSCLC patients is of around 20% compared to the non-mutated patients (Fig. 3), whose targeted therapy can be based on the levels of PD-L1/PD-1 axis that is actually clinically validated. The expression of PD-L1 can be divided in high if tissue staining is above 50%, low if the expression is between 1 and 49% and negative if there is no staining (Planchard et al., 2018). Patients who have no genetic mutation/s and that present high levels of tumor-associated PD-L1 are treated with Pembrolizumab (anti-PD-1 monoclonal antibody) as first-line, as several clinical trials showed benefits in terms of progression-free survival (PFS) and quality of life, despite data on overall survival (OS) were less promising.

In particular, data from phase 3 KEYNOTE-024 study performed on untreated advanced NSCLC patients with PD-L1 expression of at least 50% on tumor cells and no sensitizing genetic mutation, indicated that median progression-free survival was 10.3 months (95% confidence interval [CI], 6.7 to not reached) for the Pembrolizumab group versus 6.0 months (95% CI, 4.2 to 6.2) for the chemotherapy group (hazard ratio for disease progression or death, 0.50; 95% CI, 0.37 to 0.68; $P < .001$). The estimated rate of overall survival at 6 months was 80.2% for the Pembrolizumab group versus 72.4% for the chemotherapy group (hazard ratio for death, 0.60; 95% CI, 0.41 to 0.89; $P = .005$). The response rate was higher for the Pembrolizumab group than for the chemotherapy group (44.8% vs. 27.8%), the median duration of response was longer (not reached [range, 1.9+ to 14.5+ months] vs. 6.3 months [range, 2.1+ to 12.6+]), and treatment-related adverse events of any grade were less frequent (occurring in 73.4% vs. 90.0% of patients), as were grade 3, 4, or 5 treatment-related adverse events (26.6% vs. 53.3%) (Reck et al., 2016). In contrast, patients with low expression of tumor-associated PD-L1 are still treated with chemotherapy in combination with anti-PD-1/PD-L1 agents (Planchard et al., 2018). Interestingly, patients with no positive staining for this immune checkpoint are treated with the classical chemotherapy in combination with Pembrolizumab, regardless of PD-L1 status. This is due to the fact that, although at a lower dose, patients treated with Pembrolizumab and chemotherapy, still show benefits, implying a non-specific activity of the antibody or a still cellular/molecular unknown activity of it (Socinski et al., 2018). Moreover, the disappointing data on overall response rate of patients treated with immune checkpoint inhibitors could be related to recent findings by which PD-L1 is not a good prognostic biomarker. To date, a novel concept of resistance to PD-L1 blockade therapy could harbor on the two novel identified PD-L1 splicing variants which lack the transmembrane domain of PD-L1 and that once secreted can behave as decoy sequestering anti-PD-L1 antibodies

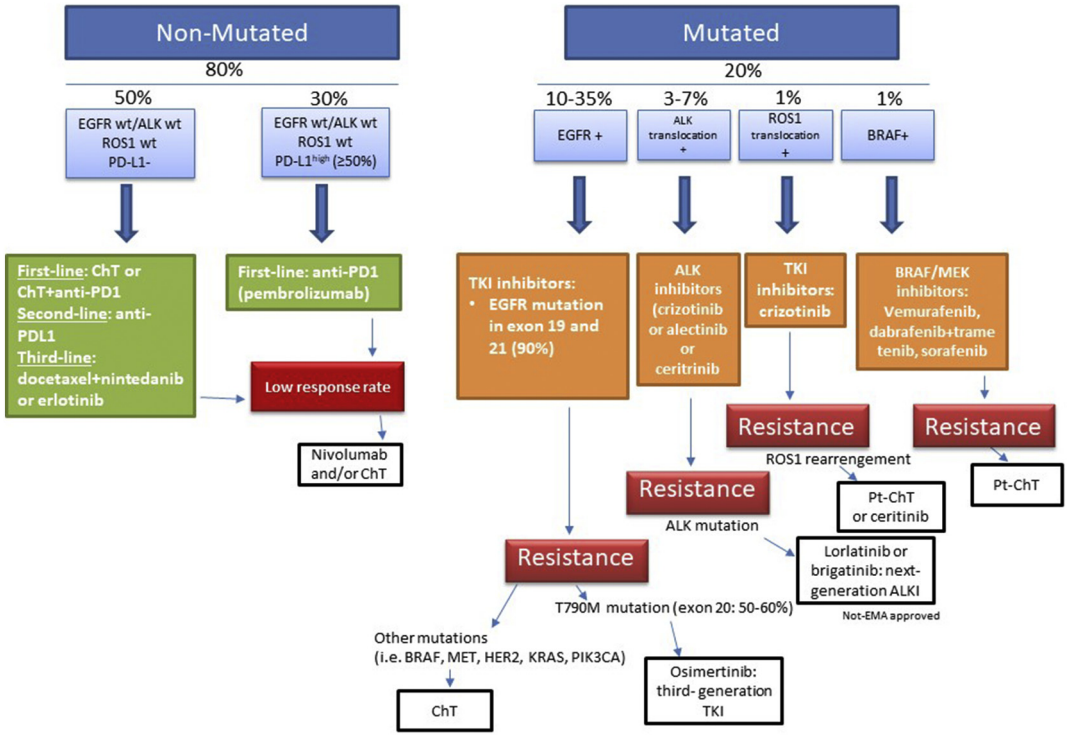

Fig. 3. Therapeutic strategy for non-mutated vs mutated NSCLC patients.

(Gong et al., 2019 JEM). However, it still remains to be elucidated the molecular mechanism/s that underlie the resistance to anti-PD-1 treatment.

To note, however, other ongoing clinical trials (Phase II) are focusing on the effect of anti-LAG-3 agents. LAG-3 is another immunesuppressive membrane molecule which can counter an effective adaptive immunity by inhibiting MHC II on dendritic cells (DCs), favoring T regulatory cells to overcome, that in the context of tumor, represents tumor progression. Data from the ongoing clinical trials will be available by 2021, but certainly open new therapeutic options, especially for non-mutated patients (https://clinicaltrials.gov/ct2/show/NCT03625323). Concomitantly, a recent retrospective study found LAG-3 over-expressed on tumor-infiltrating lymphocytes (TILs) in non-adenocarcinoma NSCLC patients, correlated to PD-1/PD-L1 expression (He et al., 2017). Recurrence-free survival was significantly different in patients whose TILs were LAG-3-negative as opposed to LAG-3-positive (1.91 years [95% CI: 0.76–3.06] versus 0.87 years [95% CI: 0.27–1.47] [p = .025]). Likewise, LAG-3 status on TILs (negative versus positive) did significantly affect overall survival (OS) (3.04 years [95% CI: 2.76–3.32] versus 1.08 years [95% CI: 0.42–1.74] [p = .039]). Moreover, patients with both PD-L1-negative tumor cells and LAG-3-negative TILs had longer recurrence-free survival than patients who were either PD-L1- or LAG-3-positive or both PD-L1- and LAG-3-positive (2.09 years [95% CI: 0.90–3.28] versus 1.42 years [95% CI: 0.46–2.34] versus 0.67 years [95% CI: 0.00–1.45] [p = .007]). Higher expression of LAG-3 was also significantly correlated with higher expression of PD-1 on TILs (p = .016) and PD-L1 on tumor cells (p = .014). Interestingly, there was no correlation between LAG-3 expression and EGFR (p = .325) and/or KRAS mutation (p = 1.000) or ALK fusion (p = .562) (He et al., 2017). Nevertheless, the synergistic interactions between various immune checkpoints requires further exploration, in order to be able to plan an effective treatment strategy that can contrast the progression of the pathology.

2.1. Target therapy plus immunotherapy: Benefits or downside for non-mutated patients?

Despite lung cancer has always been considered poorly immunogenic, the recent development of immune checkpoint inhibitors (CPIs) has upended this belief and provided proof of principle that immunotherapy can play an important role in the treatment of patients with lung cancer. In NSCLC, therapies with an antibody targeting PD-1/PD-L1 and CTLA4, another immunesuppressive mediator, represent a remarkable advance in lung cancer treatment and demonstrated response rates of 17 to 21%, with some response being remarkably durable (Rizvi et al., 2015).

In the last three years, US Food and Drug Administration (FDA) approved drugs targeting PD-1 pathway (Nivolumab, Pembrolizumab, and Atezolizumab) in both chemotherapy-naïve and previously treated advanced stage non-mutated NSCLC patients. Phase III KEYNOTE-024 clinical study has established the role for Pembrolizumab as first-line treatment in patients with untreated, advanced NSCLC and tumor characterized by PD-L1 expression ≥50%, in the absence of EGFR or ALK alterations (Planchard et al., 2018; Reck et al., 2016). Indeed, tumors with high infiltration of T cells may demonstrate higher PD-L1 expression as a form of adaptive resistance mechanism and are more likely to benefit from PD-1/PD-L1 inhibition. While most studies concur that higher tumor-associated PD-L1 expression is correlated to improved clinical outcome/response to PD-1/PD-L1 blockade, there is evidence that a subpopulation of patients with PD-L1-negative tumors may also have clinical benefit from immune CPIs (Planchard et al., 2018). Nevertheless, the immune checkpoint blockade has become part of the standard-of-care treatment option for patients with advanced stage NSCLC, although only a small subset (20–30%) of patients responds to the treatment (Jain, Jain, & Velcheti, 2018). Because immune CPIs work by reactivating immune responses, FDA approved them in combination with chemotherapy (e.g. Pembrolizumab plus Carboplatin/Pemetrexed), due to an improved overall response rate (ORR) (Fig. 3). Although response rate and PFS were increased when Pembrolizumab was added to chemotherapy, overall survival (OS) remained unchanged (Jain et al., 2018). Thus, there is a growing need to identify predictive and prognostic biomarkers for better selection of patients in order to perform a targeted therapy.

Another diagnostic/prognostic tool that has been recently introduced is the Tumor Mutational Burden (TMB) which defines patients as high or low TMB-related according to the cut-off mutation/genetic aberrations. Initially, TMB has shown encouraging results as a predictive biomarker in NSCLC retrospective studies; nevertheless, there is no statistical correlation between PD-1/PD-L1 expression and TMB related to the patient prognosis/overall survival. Although immune CPIs are broadly efficacious, improved outcomes have been observed in patients with high PD-L1 expression or high TMB (Gandara et al., 2018). Blood-based TMB reproducibly identifies patients who derive clinically significant improvements in PFS from Atezolizumab (anti-PD-L1) in second-line and advanced NSCLC (Gandara et al., 2018). In phase III trial CheckMate 227, advanced NSCLC patients with high TMB (≥10 mutation/Mb) receiving Nivolumab (anti-PD-1)/Ipilimumab (anti-CTLA-4) presented longer PFS (three-fold higher than 1-year PFS; 42.6% versus 13.2%) compared to chemotherapy treatment (Planchard et al., 2018). PFS benefit with Nivolumab/Ipilimumab was seen regardless of PD-L1 tumor proportion score (TPS) (Planchard et al., 2018). Rizvi and colleagues (Rizvi et al., 2015) showed that in NSCLC patients treated with Pembrolizumab (anti-PD-1), higher somatic non-synonymous mutation (especially C-to-A transversions) burden strongly associated with clinical efficacy, higher ORR and PFS. In particular, clinical efficacy correlated with a molecular signature typical of tobacco carcinogen-related mutagenesis, certain DNA repair mutations (POLD1, POLE, MSH2 gene), and burden of neoantigens such as HERC1 P3278S (ASNASSAAK) (Rizvi et al., 2015).

NSCLC mutation load can affect tumor immunogenicity. Thus, targeted therapy can enhance the antitumor immune responses by releasing new antigens; this provides a theoretical basis for immunotherapy combined with targeted therapy. At present, immunotherapy combined with targeted therapy in NSCLC patients is still at an immature phase, and its effectiveness and safety has mainly been assessed by preclinical studies and early clinical trials.

In order to improve clinical outcomes in patients with NSCLC harboring EGFR mutations, accompanied by up-regulation of PD-L1 expression, several in vitro studies have been conducted demonstrating that high expression of PD-L1 was associated to EGFR mutation and therefore, EGFR-TKIs can down-regulate PD-L1 resulting in an enhanced antigenicity (Akbay et al., 2013; Azuma et al., 2014), supporting the efficacy of immune−/target-therapy combination. Despite these stimulating results, obtained from in vitro studies or animal models, in clinical practice, EGFR-mutated NSCLC patients often lack TILs (Mazzaschi et al., 2018). Several clinical trials are in progress to examine the combined effects of EGFR-TKIs and immune checkpoint inhibitors, but the results obtained so far appear to be conflicting with a predominance of evidence of failure of synergistic tumor cell-killing effect (Liang, Liu, & Wang, 2018). Overall, tumor immune escape occurs in EGFR-mutated NSCLC patients via the up-regulation of PD-L1 expression; thus, EGFR-TKIs and PD-1 monoclonal antibodies may have additive but not synergistic therapeutic effects in targeting this mechanism.

3. Target therapy and the hurdle of the acquired resistance

TKIs directed at sensitizing mutations in EGFR and ALK genes represent a critical pillar in NSCLC treatment and are the standard-of-care for mutated NSCLC patients who have a better response rate and PFS compared to chemotherapy. Unfortunately, despite the excellent disease control in the initial stage of the therapy, targeted therapy fails to prolong the OS of these patients. This paradox may be attributed to the

loss of responsiveness. Indeed, almost all patients develop acquired resistance limiting the duration of the clinical benefits and reducing the mean PFS range to 9.2–13.1 months (El Kadi et al., 2018). Resistance to targeted therapies is generally classified as either primary (i.e. intrinsic) or secondary (i.e. acquired) (Gainor & Shaw, 2013). Primary resistance describes a de novo lack of treatment response, whereas acquired resistance is defined as progression after initial benefit. This latter is typical of EGFR/ALK-mutated NSCLC patients who achieve an effective response or are in a stable disease condition for longer than six months, but subsequently develop disease progression while they are still on the targeted therapy (Jackman et al., 2010). The most common resistance mechanism is a single-nucleotide transition mutation in EGFR; in particular, a cytosine to thymine (C > T) at position 2369 (CAC/CAT) causing threonine to methionine aminoacid change at codon 790 (T790 M) (El Kadi et al., 2018). T790 M was found in about 50% of EGFR–mutated cases that acquired resistance to either first- or second-generation EGFR-TKIs. It has to be pointed out that some studies reported that high T790 M positivity detected in TKI naive NSCLC patients may in some cases be formalin-fixed paraffin-embedded (FFPE)-derived artifacts (Ye et al., 2013). Indeed, T790 M mutations were detected in formalin-fixed cancer specimens, but not in their matched fresh frozen samples, suggesting that care should be taken when analyzing FFPE material to significantly reduce or eliminate artificial errors. However, in the recent years the search for new specific and sensitive mutation assays is making great strides, minimizing the error rate.

The 790 residue of EGFR is in a key location, at the level of the hydrophobic pocket for ATP-binding cleft, which causes a conformational change that leads to the development of steric hindrance, avoiding EGFR-TKI to bind the ATP-kinase pocket. In addition, the T790 M mutation of EGFR could restore the affinity of the mutant receptor for ATP, thus reducing the potency of competitive inhibitors.

Other second-point mutations (Table 1), such as D761Y, T854A, or L747S, confer acquired EGFR-TKI resistance, although the definite mechanism is still unclear.

In a recent report, El Kadi and colleagues proposed a new mechanism driving T790 M mutation (El Kadi et al., 2018). They suggest that the threonine/methionine aminoacid change is caused by the deamination of the 5-methylcytosine to thymidine at position c.2369 mediated by the activation-induced cytosine deaminase (AICDA), an enzyme induced after treatment with first-, second-, and third-generation EGFR-TKIs (El Kadi et al., 2018). Indeed, knocking down AICDA in a pharmacological manner through the inhibition of NF-κB pathway or in a genetic manner through CRISPR technique, avoided T790 M development after TKI exposure. Therefore, targeting AICDA activity directly (developing specific inhibitors) or indirectly with NF-κB pathway inhibitors might lead to the delay or prevention of T790 M-related resistance o TKIs. The evaluation of EGFR T790 M mutation is pivotal to define an acquired resistance as it can inform about the selection of future therapeutic options. To date, a number of third-generation TKIs are under development, but Osimertinib is the only agent approved by both FDA and EMA for the treatment of advanced EGFR T790 M mutation-positive NSCLC patients, based on the impressive data from Phase I/II AURA, Phase II AURA2 and Phase III AURA3 studies (Girard, 2018). Therefore, Osimertinib represents the current standard therapy for the treatment of 50%–60% of EGFR-mutated NSCLC patients who develop T790 M mutation on progression with Erlotinib, Gefitinib, or Afatinib (Westover, Zugazagoitia, Cho, Lovly, & Paz-Ares, 2018). Osimertinib significantly prolongs PFS versus chemotherapy (10.1 vs 4.4 months; HR: 0.30; 95% CI: 0.23–0.41; $p < .001$) and is associated with a significantly higher response rate (71 vs 31%; odds ratio [OR]: 5.39; 95% CI: 3.47–8.48; $p < .001$), although OS data have not been reported yet (Girard, 2018).

While EGFR T790 M mutation is essentially the sole, clinically predominant resistance mechanism after failure of first- and second-generation EGFR-TKIs, for ALK, numerous kinase domain mutations (Table 1) have been reported in patients with acquired TKI resistance. The ALK-L1196 M gatekeeper mutation confers resistance through steric interference. Other 'not-gatekeeper' second-site ALK mutations are distributed throughout the kinase domain, including the solvent front (G1202R, S1206Y), ATP binding pocket (G1269A), and N-terminal to the C-helix (1151Tins, L1152R and C1156Y) (Muller, De Langen, Honeywell, Giovannetti, & Peters, 2016).

Treatment with the first generation ALK inhibitor Crizotinib is more effective than standard chemotherapy. However, resistance to Crizotinib occurs after approximately 8 months. Ceritinib is the first second-generation ALK inhibitor approved for treatment of Crizotinib-resistant NSCLC. Ceritinib inhibits two of the most common ALK-mutants that confer resistance to crizotinib: L1196 M and G1269A (Muller et al., 2016).

Alternatively, following the development of additional genetic alterations in the primary oncogene, which facilitates continued downstream signaling, resistance can develop independently of genetic changes in the target, through up-regulation of bypass signaling pathways, changes in tumor histology or alterations in drug metabolism. About that, in another interesting study, it has been proposed a strategy to sensitize NSCLC cells to EGFR-TKIs, through the inhibition of the aryl hydrocarbon receptor (AhR), identified as an EGFR-TKIs resistance mediator, not associated to mutation and independently of its canonical transcriptional activity (Ye et al., 2018). In particular, the activation of AhR signaling led to conformation changes of the AhR protein, resulting in enhanced binding with Src kinase. AhR transiently translocates to the cell membrane and acts as an adaptor to mediate the cross-talk between Jak2 and Src, bypassing mutant EGFR to restore PI3K/Akt and MEK/Erk signaling and eliciting a TKI-resistant phenotype (Ye et al., 2018).

Although there is no direct evidence showing that the activation of AhR leads to lung tumorigenesis, overexpression of AhR has been detected in >50% of NSCLC patients and polymorphisms of AhR gene are implicated in the development of the pathology. Activation of AhR signaling up-regulates several growth factors and it is plausible that AhR is involved in determining the sensitivity of NSCLC to apoptosis-inducing agents, such as targeted therapeutics.

In addition to the mechanism just described, among 'bypass' resistance mechanisms that utilize alternative cellular pathways to activate the same key downstream effectors of tumor cell growth and survival, the most common, for resistance to first- and second-generation EGFR-TKIs is the amplification of ERBB2, a gene that encodes for the ErbB family member HER2 (Westover et al., 2018). ERBB2 amplification occurs in 10%–15% of patients with EGFR TKI resistance, followed by the amplification of the MET gene, which encodes MET tyrosine kinase receptor, reported in 5% of patients. MET is activated by hepatocyte growth factor, thereby potentiating growth, survival, and invasion pathways via Src, PI3K, and RAS family members (Westover et al., 2018). Although there are no targeted therapies specifically approved for patients with EGFR-mutated NSCLC patients who progress on EGFR-TKI due to MET amplification, two phase Ib/II trials of the MET inhibitor Capmatinib (INC280) alone or in combination with Erlotinib (NCT02468661) and Gefitinib (NCT01610336) are currently under way (Westover et al., 2018).

Other Phase I studies are assessing the combination of Osimertinib with the VEGFR2 antibody, Ramucirumab, or the EGFR antibody, Necitumumab, in patients with T790 M-positive NSCLC (NCT02789345) and the combination of Osimertinib with Navitoclax, a BCL-2 family inhibitor (NCT02520778) (Girard, 2018).

Besides, a recent study provides proof-of-concept that RET fusions can mediate acquired resistance to TKIs in EGFR-mutated NSCLC patients, and that the combination of Osimertinib with BLU-667 (potent and selective inhibitor of RET alterations) has the potential to become an important tool to overcome and prevent the emergence of clinical resistance. BLU-667 has been shown to be >15 times more potent on RET than any other kinase and > 10 times more potent on RET than approved multi-targeted kinase inhibitors like Cabozantinib (Piotrowska et al., 2018).

Other 'bypass' mutations have been reported at a lower frequency; these include mutations in BRAF, PIK3CA, KRAS, PTEN loss, NF-1 loss, and CRKL amplification. Furthermore, resistance mediated by non-genetic changes has also been described, including up-regulation of IGF1R, FGFR, hepatocyte growth factor, and the ligand of MET. Likewise, activation of the receptor tyrosine kinase AXL is associated with resistance to EGFR TKIs (Westover et al., 2018).

Therefore, gaining insight into the mechanisms underlying tumor cell drug resistance is critical for developing more effective therapies, since to date, much more is known, but a fixed point has not yet been put and a certainty for resistant NSCLC patients is still missing.

4. Detection of mutation/s to improve target therapy: A step forward but others to be done

Because the tumors in different patients may acquire resistance via differential mechanisms, it is important to evaluate each patient's unique mechanism of resistance at the molecular level in order to tailor targeted therapies to individual patients. Originally, re-biopsy of tumor tissue was the only option for collecting tumor material to assess acquired mutation (i.e. T790 M status), though this method required high level of patient compliance and was rather invasive. Moreover, biopsy may not always be feasible due to accessibility, and even if it is, the procedure is not without risk. Indeed it is associated with a failure rate of 5–10%, despite availability of sufficient tissue, not reflecting differences in tumors across sites (Girard, 2018). In addition, scheduling the biopsy and performing testing can ultimately delay subsequent therapy. Recently, the focus of precision oncology is increasingly turning to liquid biopsy as it is becoming an increasingly attractive alternative to tissue re-biopsy. Liquid biopsy offers a number of potential improvements over tissue biopsy, including decreased cost, greater compliance (because non invasive), improved patient safety, quicker turnaround time. Moreover, blood-based monitoring of disease progression represents a safer and faster way to make treatment decisions compared to tissue biopsy (Heitzer et al., 2018).

A post hoc analysis of AURA showed that sensitivity of plasma genotyping for EGFR T790 M was 70% in patients who were T790 M-positive on central tumor genotyping. However, some patients had positive T790 M on tissue but not on liquid biopsy, related to the lower sensitivity of plasma analyses. Interestingly, some patients had positive T790 M on liquid biopsies that was not observed in the tumor tissue, a situation which may reflect spatial heterogeneity, with possible impact on the efficacy of osimertinib (Girard, 2018). As such, the authors suggested that plasma genotyping could be performed as an initial step to detect T790 M mutations, and that patients who are T790 M-positive by using plasma, could proceed with third-generation TKI therapy without biopsy. However, those patients with T790 M-negative results in plasma-derived samples should perform tumor biopsy to confirm T790 M-negativity/positivity and determine subsequent therapy (Girard, 2018). Cobas EGFR Mutation test v2 is a circulating cell-free DNA (cfDNA) test approved by FDA to detect EGFR mutation (Heitzer et al., 2018). Another promising analyte for precision oncology application is the circulating tumor DNA (ctDNA), which analysis enables to detect resistance markers (i.e. T790 M) in lung cancer patients (Heitzer et al., 2018) (Fig. 1). Despite many benefits, substantial limitations for liquid biopsy are represented by 1. low levels of the circulating analyte (cfDNA, ctDNA or CTC), 2.confounding factors resulting from ageing, 3. validation issues, 4. special equipment required for the detection and/or isolation, 5. high degree of heterogeneity, 6. no high-throughput procedures available, 7. high variable tumor fractions even in advanced stages, 8. intrinsic instability (i.e. circulating cell-free RNA) and 9. unknown composition (i.e. extracellular vesicles) of some analytes (Heitzer et al., 2018). A new strategy applicable to liquid biopsy in lung cancer, is the hybrid capture (HC)-based next generation sequencing (NGS), able to reveal more actionable genomic alterations than do standard diagnostic methods. It offers broad gene sequencing and provides extensive genetic information regarding the broad aberration repertoire, including information on exon and intron mutations, gene rearrangement and amplifications, providing a key for therapeutic decision making (Rozenblum et al., 2017). A recent study demonstrated that in addition to standard molecular testing (for EGFR/ALK mutation), HC-based NGS resulted in a changed treatment strategy for 37% of NSCLC patients and was associated with an ORR of 65%, which may translate into a survival benefit (Rozenblum et al., 2017).

Despite the diagnostic landscape in NSCLC is evolving rapidly, with new tumor profiling tests becoming available every year, many challenges need to be solved to obtain clinical validity and utility in order to reach the expected goal. To date, the financial aspect cannot be underestimated: the cost of one assay of tissue/liquid HC-based NGS in 2015 was $5274 and $4838, respectively (Rozenblum et al., 2017).

5. Conclusions

Key established predictive biomarkers for target therapy include ALK rearrangements, ROS1 rearrangements, sensitizing EGFR mutations, BRAF V600E point mutations, and PD-L1 expression levels. The National Comprehensive Cancer Network (NCCN) panel recommends testing for these key established biomarkers in patients with advanced NSCLC before initial treatment, because effective targeted therapy or immunotherapy is available.

However, acquired resistance to targeted therapies represents a major clinical challenge in the treatment of NSCLC, a disease with high molecular complexity, in that understanding deeper resistance mechanisms has become an urgent imperative.

Advances in the detection method for different resistance mechanisms and the development of new drugs are both urgently needed for personalized therapy, especially for non-mutated patients. Moreover, newer genomic approaches are needed to understand the entire drug-resistance repertoire for better treatment of lung cancer subjects in a more personalized manner. Many avenues combating treatment resistance in lung cancer patients are currently being developed and explored, leading to a promising future for the advancement of molecular targeted therapy for NSCLC patients.

Unfortunately, though, non-mutated patients have limited therapeutic options other than chemotherapy, which is no longer a standard, and immune CPIs, despite PD-L1 score. Therefore, it appears as a default if patients do not have an actionable oncogenic driver.

In conclusion, it is therefore becoming more and more evident that selecting the optimal first-line treatment requires consideration of patient factors, diagnostic/prognostic tools, diagnostic tools to highlight potential mechanism/s of drug activity and resistance, in order to subsequently have therapy options for long-term treatment and the related tolerability profile according to the overall survival of the patient.

Declarations of Competing Interest

There is no conflict of interest to disclose.

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