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REVIEW

The role of molecular heterogeneity targeting resistance mechanisms to lung cancer therapies

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ABSTRACT

Introduction: The treatment scenario of lung cancer is rapidly evolving through time. In parallel, growing evidence is accumulating on different mechanisms of treatment resistance. Inter- and intra-tumor heterogeneity define the spatial and temporal tumor clonal evolution, that is at the basis of tumor progression and resistance to anticancer treatments.

Areas covered: This review summarizes the available evidence on molecular heterogeneity in lung cancer, from diagnosis to the occurrence of treatment resistance. The application of novel molecular diagnostic methods to detect molecular heterogeneity, and the implications of understanding heterogeneity for drug development strategies are discussed, with focus on clinical relevance and impact on patients' survival.

Expert opinion: The current knowledge of molecular heterogeneity allows to identify different molecular subgroups of patients within the same conventional tumor type. Deeper understanding of heterogeneity determinants and the possibility to comprehensively investigate tumor molecular patterns will lead to the development of personalized treatment approaches, with the final goal to overcome resistance and prolong survival in lung cancer patients.

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Clonality; next-generation sequencing; resistance; tumor evolution; tyrosine kinase inhibitors

1. Introduction

In the last decades, a progressive reduction in overall lung cancer mortality has been observed [1]. This is only in part related to the reduction of lung cancer incidence and improvements in early diagnosis. Indeed, the recent advances obtained in the field of lung cancer treatment led to nearly 10% increase in 5-year relative survival from 1980s to present time [1]. The main achievements concern target treatments (e.g. selective tyrosine kinase inhibitors for tumors harboring driver gene alterations) and immunotherapy, which were able to determine durable responses and long-term survival in the setting of advanced disease [2,3]. However, lung cancer remains the leading cause of cancer death, as most patients inevitably develop resistance to treatments [1,4–6].

Regardless of the specific treatment adopted, resistance can occur either as a primary or as an acquired mechanism, and is closely related to tumor heterogeneity [7,8]. Indeed, the survival and progression of cancer cells are based on evolutionary selective pressure deriving from intrinsic and extrinsic factors (including the tumor microenvironment and anticancer treatments), determining the co-existence of different tumor clones or subclones across tumor regions (spatial heterogeneity) and also dynamically over time (temporal heterogeneity) [9]. This concept applies to the overall complex ecosystem involving both cancer cells and nonmalignant cells (e.g. immune-stromal compartment) and occurs either at genetic or non-genetic (i.e. epigenetic, transcriptional) levels [8] (Figure 1).

The aim of this review is to comprehensively address the issue of molecular heterogeneity as a determinant of treatment resistance in lung cancer, focusing on its implications from the moment of molecular diagnosis, to the applications in drug development and approach to resistance, with the final goal to understand how to integrate the knowledge of heterogeneity into the clinical setting.

2. Body

2.1. Exploring the drug selective pressure on tumor clonal evolution

The emergence of new targeted therapies in recent years improved the survival rates in non-small cell lung cancer

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Article highlights

- Tumor clonal evolution is at the basis of cancer progression and adaptation to anticancer treatments
- Molecular heterogeneity is responsible for both inter- and intra-tumor variability
- Novel diagnostic techniques allow to detect molecular heterogeneity at different steps of tumor evolution
- Personalized treatment strategies based on deeper knowledge of tumor molecular heterogeneity might represent the way forward to overcome treatment resistance in lung cancer

(NSCLC) patients [10]. However, primary or secondary resistance to treatment arise in the majority of patients, remaining a challenge to overcome [11]. Primary resistance is generally caused by the presence of intrinsic mechanisms in tumor cells preexisting at the beginning of treatment. Secondary resistance arises in tumor cells that are initially responsive to therapy, and become insensitive during treatment [12,13]. The characterization of tumor heterogeneity may be a starting point for the elucidation of the possible mechanisms at the basis of resistance to treatments.

It is well known that tumors showing the same stage or histological characteristics are carriers of different molecular clones, which may be associated to a different clinical response. Therefore, it is important to understand how therapies influence the survival of each clone, considering that drugs are able to differentially affect the death or survival of different tumor clones, leading to the proliferation of resistant clones [14,15]. Considering tumors harboring 'druggable' drivers, arising mechanisms of resistance are usually linked to the driver itself, depending on the drug potency. In particular, drugs with high potency on their target may generate mechanisms of resistance linked to the activation of signaling pathways independent from the driver. Due to its dynamic changes, cancer is considered as a mixture of complex clones, with different characteristics and response to treatment. Recently, the concept of 'drug-tolerance' emerged as a link between tumor heterogeneity and the microenvironment [16]. It has been demonstrated that specific subpopulations, called 'Drug-Tolerant Persisters' (DTPs), can survive also in the presence of high-dose therapy. Interestingly, these sub-clones harbor specific markers of stem cells and can change their phenotype under the selective pressure of therapy [16]. DTPs, de novo mutations and preexisting resistance are considering some of the possible ways in which cancers evade the anticancer drug pressure (Figure 1). Today the challenge is to delay the emergence of these mechanisms, using new target drugs or new dosing/schedules strategies.

3. The role of different molecular diagnostic techniques in understanding tumor heterogeneity

Several factors may impact on tumor heterogeneity as a considerable mechanism able to predict clinical response in lung cancer patients [17–20]. Among them, mutant allele specific imbalance (MASI) indicates the differential expression of mutated with respect to wild type allele fraction by influencing tumorigenesis, progression, metastasis, prognosis and potentially therapeutic responses in cancer [21]. Previous studies showed that MASI occurred in several driver oncogenes (*EGFR, KRAS, PIK3CA*, and *BRAF*) in a large cohort of patients with solid tumors [21]. Sanger sequencing was the first approach used to inspect MASI in tumor diagnostic [22].

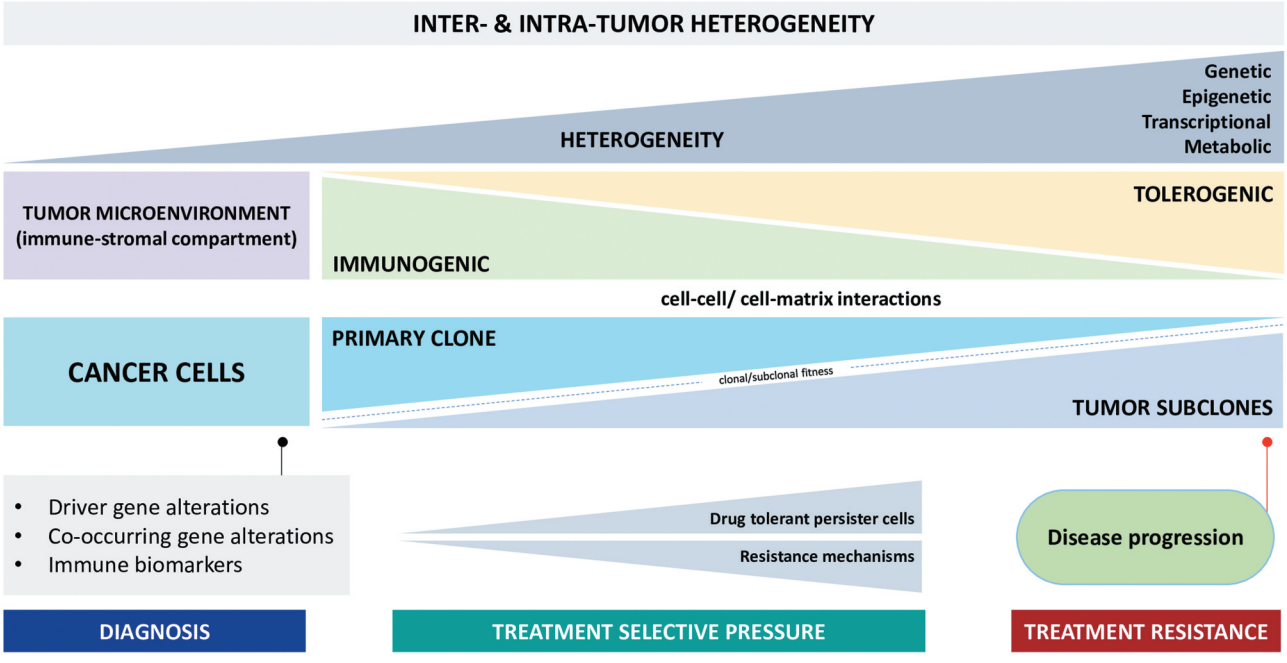

Figure 1. Principles and evolution of inter- and intra-tumor heterogeneity tumor heterogeneity (upper panel) increases as the result of a complex eco-system, involving cancer cells and tumor immune-stromal compartment. these two components dynamically and temporally interact (middle panel), from the first steps of clonal evolution and under the treatment selective pressure, finally resulting into disease progression (lower panel) as a consequence of increased tumor clonality and immune tolerogenicity.

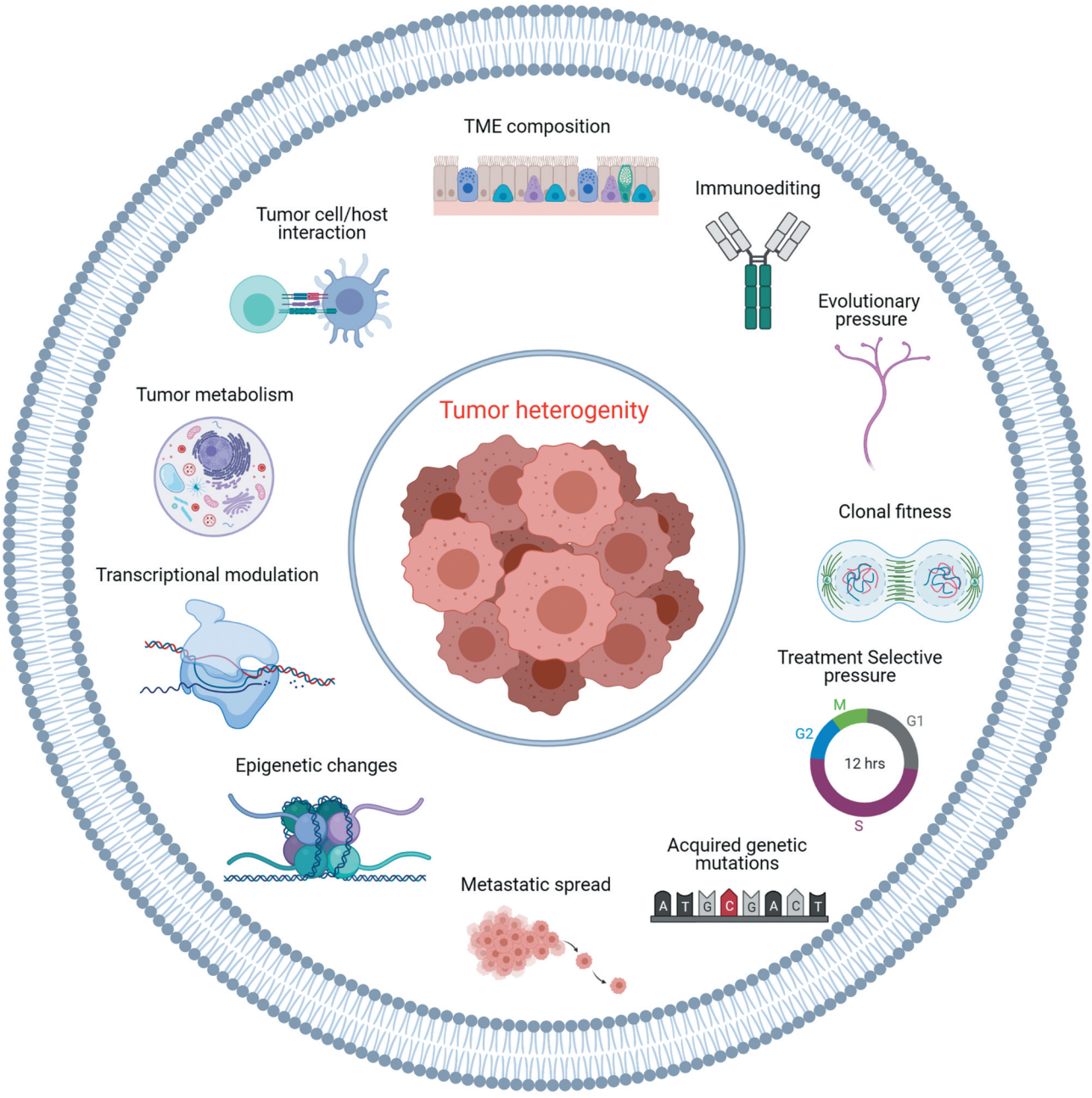

Figure 2. **Hallmarks of tumor heterogeneity** The ring figure graphically summarizes the major determinants of tumor heterogeneity: intrinsic tumor cell processes, selective pressure, tumor and immune microenvironment and tumor/host interaction contribute to the evolution of tumor heterogeneity, both as single factors and as interacting and dynamic phenomena.

Chen *et al*, used a direct sequencing platform for the inspection of *EGFR* heterogeneity in 180 pairs of lung adenocarcinoma samples and corresponding metastatic sites. It was found a high discordant rate in *EGFR* mutations (13.9%), and it was observed that clinical response to tyrosine kinase inhibitors (TKIs) is influenced by the presence of heterogeneous tumor cells population [23]. However, conventional Sanger sequencing platform revealed several limitations for the molecular profiling of low distribution mutated cell population, in particular related to the low sensitivity of this techniques that may lead to false negative results [24]. On this basis, Jiang *et al*. analyzed pre- and post- therapeutic tissue specimens of six NSCLC patients harboring mutations under gefitinib treatment. A conventional RT-qPCR based sequencing approach (ABI PRISM 3100 genetic analyzer, Applied Biosystems) was adopted. Results showed that sensitive *EGFR* mutations (exon 19 deletion p.747-S752del and exon 21 p. L861Q point mutation, respectively) were not detected in post-treatment samples. Interestingly, a high sensitive mutant-enriched peptide-nucleic-acid (PNA)-mediated PCR clamping allowed to identify *EGFR* sensitive mutation only in one instance but it failed to correctly report p.L861Q *EGFR* point mutation [25]. Hence, microdissection-based cell cluster mutation analysis was also performed on pretreatment tumors, and heterogeneous distribution of *EGFR* mutations was inspected in 3 patients. In details, tumor cells harboring

EGFR mutations represented the minor fraction in the previous described cases, suggesting that *EGFR* clonal heterogeneity requires high sensitive methods to establish the best therapeutic choice in NSCLC patients with minor mutated cell fraction [25]. However, despite a high sensitivity targeted-based approaches, including RT-qPCR, suffer from significant limitations. These include a limited reference range, in fact these methodologies are able to detect only known gene alterations, and the limited multiplexing power. In the last decade, the impressive number of predictive biomarkers approved for NSCLC patients changed the analytical paradigm for the identification of clinically relevant mutations [26]. Therefore, multiplexing platforms were progressively implemented in routine practice in order to analyze a large series of actionable mutations that play a crucial role in the patient's clinical stratification [27]. In this scenario, next generation sequencing (NGS) is rapidly emerging as a useful technical approach able to simultaneously analyze several target genes from multiple tumor tissue in a single run. Interestingly, this approach allows to detect a wide range of clinically relevant known and unknown alterations, within the adopted panel, at low frequency by identifying small fractions of actionable mutations that may benefit from targeted treatment in NSCLC patients [27,28]. Malapelle *et al* compared two technical approaches (Sanger Sequencing platform and NGS system) for the detection of *KRAS* MASI on a large series of colorectal cancer (CRC) tissue. Results showed an unbalance between wild type and tumor allele in 12.8% (58/436) cases; interestingly, p.G13D point mutation was most frequently heterogeneously distributed in the analyzed samples [29]. By considering histological classification, Dietz *et al* investigated the role of spatial distribution of mutated allele fraction of *KRAS* and *EGFR* genes in lung adenocarcinoma specimens in relation to differential morphological features [30]. They used a custom NGS panel (LCPv2) able to identify 42 clinically relevant hot spot regions for NSCLC patients by adopting ion Torrent PGM™ System (Thermo Fisher Scientific, Hilderan, Germany). Results showed similar variant allele fraction (VAF) for *KRAS* and *EGFR* genes according to inter-tumoral spatial analysis of neoplastic area, while significant variation in terms of VAF was observed with respect to predominant tumor growth pattern [30]. Another crucial issue related to tumor molecular heterogeneity depends on the partial comprehension of a plethora of molecular alterations that could derive a worse clinical outcome in patients receiving TKIs. Lee *et al*, approached NGS analysis by using an extensive NGS panel (AmpliSeq Comprehensive Cancer Panel, 409 genes) on *EGFR* mutant NSCLC patients by comparing mutation profile of pre-treatment formalin fixed paraffin embedded (FFPE) samples with corresponding tissue specimens collected at progression on first-line TKI [31]. The authors highlighted that p.T790M exon 20-point mutation was the most common acquired resistance mutations (63.2% of cases) but in a not negligible number of patients the identification of novel molecular signature could predict clinical outcome in NSCLC patients [31]. Liquid biopsy is recommended for the analysis of sensitive *EGFR* mutations when tissue is not available at primary diagnosis or to detect acquired resistance mutation after disease progression on TKI [32]. Indeed, cell-free DNA (cfDNA) analysis allows to overcome the spatial limit of tissue specimens in detecting tumor heterogeneity, but the analysis of circulating tumor DNA (ctDNA), that represents a small fraction of cfDNA, underlines the need for implementation of ultra-deep technologies to correctly perform molecular [33,34]. The identification of specific molecular signatures may also play a pivotal role in the patient stratification at early-stage setting. In this scenario, RNA single cell analysis (scRNAseq) revealed the activation of molecular programs that impact on the clinical response of tumor cells [35]. Maynard *et al* showed that scRNAseq analysis of NSCLC patients at various clinical stages highlighted new recurrent molecular pathways that reduce clinical response to ongoing therapy, highlighting the biological heterogeneity and evolution during systemic therapies [36]. However, despite the undoubted advantages, NGS approach requires high trained personnel and careful validation steps before the implementation in clinical routine laboratory practice.

4. Pros and cons of liquid biopsy to evaluate the molecular heterogeneity in resistance to lung cancer therapies

Despite the significant improvements in molecular approaches for clinically relevant biomarkers assessment, a not negligible percentage (up to 30%) of advanced stage NSCLC patients do not have adequate tissue specimens for molecular analysis [37–39]. In this setting, liquid biopsy represents a rapid and noninvasive alternative to tissue biopsy ensuring the molecular assessment of clinical relevant biomarkers in advanced stage NSCLC patients in order to avoid to leave any patient behind [40]. Currently, ctDNA extracted from plasma has been approved by Food and Drug Administration (FDA) to evaluate the *EGFR* gene assessment in advanced stage NSCLC patients at diagnosis with unavailable tumor tissue or inadequate molecular results (basal setting) on tissue, and at progression after first- or second-generation EGFR TKIs to evaluate the presence of the *EGFR* exon 20 p.T790M resistance point mutation (resistance setting) [37–39]. To date, the number of clinical applications for liquid biopsy are rapidly growing. Among these, liquid biopsy may play a relevant role to better define the heterogeneous genomic landscape of advanced stage NSCLC patients which can change during therapy administration [32,41]. In this setting, liquid biopsy may be a useful tool to overcome the limitation of tissue biopsies to evaluate this heterogeneous scenario [32,42–44]. In fact, liquid biopsy may be adopted to monitor tumor evolution due to its minimally invasive nature and repeatability respect to tissue biopsies [32,43]. In addition, liquid biopsy can avoid sampling bias related to the presence of different distant metastatic lesions, ensuring the possibility to detect as rapid as possible emerging resistance mechanisms during treatment [32]. In fact, regardless of the site and the number of metastasis, cancer DNA is actively (spontaneous release) or passively (necrosis or apoptosis) released into the bloodstream [45]. Thus, ctDNA analysis represents a valid solution to evaluate the clonal evolution and better clarify the heterogeneity of resistance mechanisms that may arise after therapy administration [46–48]. As an example, to better assess the heterogeneity of resistance mechanisms, Chabon *et al*. adopted Cancer

Personalized Profiling by deep Sequencing (CAPP-Seq), a high sensitive NGS approach, on ctDNA extracted from 115 serial plasma samples of 43 NSCLC patients treated with the third generation EGFR TKI rociletinib. Of note, the authors highlighted a high intra-patient heterogeneity with a significant percentage of patients (46%) featuring multiple resistance mechanisms [47]. However, several limitations may affect the adoption of liquid biopsy. First of all, the risk to miss clinically relevant molecular information may be due to pre-analytical issues, including ctDNA short half-life (about 15 minutes) and very low concentration into the bloodstream (<0.5% of the total circulating cell free DNA) [38,49]. Thus, it is crucial to correctly define the time of blood sampling and to adopt highly sensitive techniques, such as NGS, to avoid 'false negative' results [38,49,50]. Another possible issue related to 'false negative' results is the possibility of non-shedder tumors, in particular when specific metastatic sites (such as intracranial) are involved [51,52]. As for 'false negative,' also 'false positive' results may be considered. In this setting, clonal hematopoiesis of indeterminate potential (CHIP) may be associated with the detection of genomic alterations into the bloodstream without the presence of a pathological event [53–55]. This phenomenon is related to the asymptomatic development of blood cells derived from a single hematopoietic stem cell, presenting genomic alterations in individuals without the evidence of hematological neoplasms [56]. As a matter of facts, the CHIP phenomenon increases in advanced ages and considers different genes; the most common are *DNMT3A, ASXL1*, and *TET2* whereas low frequently *TP53, JAK2, NOTCH2, FAT3, EXT2, ERBB4, KRAS* and *ARID2* [57–60]. Interestingly, CHIP may determine false positive results evaluation when considering to adopt ctDNA analysis. In particular, Genovese et al highlighted that mutations related to CHIP have been detected in about 10% of subjects >65 years [57].

5. Principles of drug approach for targeting clonal evolution: EGFR and ALK

A significant subgroup of patients is carrier of activating mutations in the *EGFR* and are sensitive to EGFR TKIs [61,62]. Several factors have to be considered when choosing the optimal treatment strategy to use, including the efficacy and tolerability to the individual patient characteristics, treatment sequencing, the TKI target selectivity and potency, and cost issues. First-generation TKIs are reversible inhibitors of *EGFR* and are inactive against the p.T790M resistance mutation. The second-generation TKI afatinib is an irreversible inhibitor that bounds to EGFR, ErbB2, and ErbB4, showing a higher activity than first-generation TKIs against wild type and mutant *EGFR* [63,64]. The third-generation TKI osimertinib, was developed to target both the p.T790M and the *EGFR* sensitizing mutations with high potency [62,65].

Several studies compared first, second, and third generation of EGFR TKIs for progression-free survival (PFS) and overall survival (OS), for treatment naïve *EGFR* positive NSCLC patients [66–68]. Results from trials including first- and second-generation EGFR TKIs suggested that *EGFR* exon 19 deletions and p.L858R exist as separated entity clones, with distinct biologic characteristics and intracellular signaling pathways. An in vitro study demonstrated that the phosphorylation patterns of EGFR in response to epidermal growth factor is strictly dependent on the type of mutation present in the tumor [69]. Moreover, *EGFR* exon 19 deletions and p.L858R mutations lead to distinct conformational changes to EGFR, which might underlie their different sensitivities to TKIs [70]. Furthermore, the clonal selection during treatment could differ between each EGFR TKIs and might be dependent on its pharmacologic characteristics. It has been demonstrated that the most potent inhibitor of EGFR carrying only exon 19 deletions or p.L858R mutations was afatinib (IC50 range: 0.6–1 nM), followed by erlotinib (IC50 range: 5–11 nM), gefitinib (IC50 range: 7–19 nM), and osimertinib (IC50 range: 12–60 nM) [62]. The clonal selection against exon 19 deletions or p.L858R mutations lead to the rise of the gate-keeper mutation p.T790M of *EGFR*, in patients treated with first or second generations EGFR TKIs [71,72]. The p.T790M mutation reestablishes the affinity of the mutant receptor in favor of the adenosine triphosphate (ATP), decreasing the potency of TKIs [73].

The rate of p.T790M mutation is different between NSCLC patients TKI naïve and pre-treated with first- or second-generation TKIs, suggesting the clonal selection of treatments on p.T790M positive clones and an evolution model of acquired resistance [74]. Interestingly, the incidence of the p.T790M decreases accordingly to the increasing of the drug potency [62,65] of first- versus second-generation TKIs [75]. Therefore, the extended inhibitory profile of second-generation TKIs may prevent the appearance of tumor cells that persisted with first-generation TKIs, delaying the clonal expansion and the acquisition of resistance to therapy, which is possibly reflected in the observed improvements in PFS with afatinib versus the first-generation TKI, gefitinib [76]. Recently, retrospective studies highlighted the clinical benefit with sequential second and third-generation TKIs, suggesting that resistant clones positive for p.T790M mutation are homogeneous compared to those that arise during treatment with first-generation TKIs [77,78]. The survival benefit of patients receiving sequential afatinib and osimertinib was significantly higher compared to patients treated with sequential gefitinib/erlotinib and osimertinib [78]. The third-generation EGFR TKIs have been developed to contrast tumors carrying p.T790M mutations, with higher activity against it and lower activity against wild-type *EGFR* [79,80]. Moreover, the third-generation TKI osimertinib strongly inhibits the p.T790M mutation, preventing the clonal selection and expansion of preexisting cells carrying p.T790M positive.

Similarly to *EGFR* mutant patients, *ALK*-rearranged NSCLCs benefited from the introduction in the clinical use of first-, second- and third-generation ALK inhibitors. Unfortunately, all patients with *ALK*-rearranged NSCLC develop resistance to treatment, caused by the arise and selection of *ALK* mutations (on-target resistance) or *ALK* copy number gain. The central nervous system (CNS) is the first site of progression in approximately 50% of patients [81,82], probably due to the inadequate penetration into the CNS by the first-generation ALK TKI crizotinib. Second- and third-generation ALK inhibitors (ceritinib, alectinib, brigatinib, lorlatinib) are structurally different and present higher affinity for ALK (a lower IC50), a different

selectivity for various *ALK* mutants and a better CNS penetration [83]. The overall response and PFS rates range from 39% to 56% and 5.4 to 12.9 months after crizotinib treatment failure, respectively [84–87]. The structural differences between first- and second-generation ALK TKIs have been associated with a particular profile of ALK resistance mutations [88] and the diverse potency of ALK-TKIs lead to a different selection of potent resistance mutations. It has been demonstrated that half of the tumors that develop resistance to secondary-generation ALK inhibitors presented resistance mutations in *ALK*, while the other half have other mechanisms underlying resistance, including the activation of bypass signaling as *EGFR, KRAS, KIT, MET* [89–93]. It has been demonstrated that the most potent inhibitor of *ALK*-rearranged was lorlatinib (IC50: 2.7 nM), followed by brigatinib (IC50 range: 21 nM), ceritinib (IC50: 25 nM), and alectinib (IC50: 53 nM) [94]. The third-generation ALK TKI has sub-nanomolar to low nanomolar potency against ALK and retains potency against all known resistant mutants [95]. The selectivity of lorlatinib was improved by the targeting of a specific residue (p.L1198) in the ALK tyrosine kinase domain [96]. At this position, the presence of tyrosine or phenylalanine sterically interferes with drug binding. In patient-derived cell lines, lorlatinib could inhibit the growth of cells only carrying *ALK* resistance mutations with the lower of IC50 compared to other ALK inhibitors [97]. Recently, it has been demonstrated the inhibitory effect of gilteritinib, a multi-kinase inhibitor has been clinically approved for treating *FLT3*-mutant AML, on ALK-TKI–resistant single mutants and compound mutants in vitro and in vivo. Surprisingly, in vivo model showed that gilteritinib was effective on relapsed tumor and could overcome lorlatinib resistance [98]. All these results propose that a focused investigation of the structure and functional characteristics of drug targets can be used to implement the drug design strategies and lead to clinically significant estimation of drug activity, overcoming tumor heterogeneity [99,100].

6. The influence of tumor heterogeneity on treatment response

Following the huge improvements in molecular diagnostics, driver mutant NSCLCs have been the primary field of investigation on tumor clonal evolution and tumor heterogeneity. Proof of concept was the understanding that treatment with EGFR TKIs was effective only in a subgroup of NSCLC patients, that is those harboring *EGFR* mutations [101]. Afterward, evidence has emerged about the presence of growing number of distinct variants of *EGFR* mutations, with differential responses to treatment with EGFR TKIs and specific survival outcomes [102,103]. The knowledge obtained in *EGFR* mutant setting was paramount for all the subsequently identified driver-gene alterations in lung cancer, including *ALK, ROS1, RET* gene fusions, *BRAF, KRAS, HER2, MET* gene alterations [2,104]. With the advent of novel multi-gene diagnostic panels, subclonal tumor heterogeneity was deeply addressed, and differential patterns of co-occurring gene alterations were identified across virtually all driver mutant NSCLC [105]. The most frequently investigated patterns include *TP53, STK11, KEAP1* co-mutations, which were associated with worse prognosis, shorter responses to TKIs and differential outcomes with immunotherapy [106–108]. Additionally, the clinical application of liquid biopsy not only established as a diagnostic method, but also as a useful tool to monitor disease response, resistance, and outcomes to treatments [109,110].

In parallel, molecular heterogeneity plays a role even in immune-oncology. As a matter of fact, the presence of driver gene alterations, with the only clear exception of *KRAS* mutations, predicts for worse outcomes with immune checkpoint inhibitors (ICIs) monotherapy, regardless of the expression of programmed death ligand-1 (PD-L1) in NSCLC patients [111,112]. On the other hand, comprehensive genomic profiling allows to quantify tumor mutational burden (TMB), that is the total number of nonsynonymous mutations present in a tumor specimen. The role of TMB in predicting response to ICIs is still debated, and a qualitative evaluation of molecular heterogeneity is suggested to be a potential way forward improving the outcomes of immunotherapy in NSCLC [113].

Of great interest, recent studies revealed wide intra-tumor heterogeneity in small cell lung cancer (SCLC), identifying distinct molecular profiles associated with sensitivity or resistance to chemotherapy [114]. Even more intriguing, analysis at transcriptional levels showed increased ITH at the occurrence of resistance [115,116], as well as temporal subtype switching across differential genes toward immune pathway activation [117]. This latter SCLC subtype, so-called 'inflamed' was found out to be associated to significantly better survival outcomes with chemo-immunotherapy combination as first-line treatment compared to the other known dominant subtypes (ASCL1, NEUROD1, POU2F3) [117]. Taken together, these findings outline the need to consider SCLC as a heterogeneous disease, where the deep understanding of molecular heterogeneity might have a significant impact on prognosis and treatment outcomes.

7. Conclusion

Intra-tumor heterogeneity definitively impacts the clinical course of lung cancer patients. The availability of novel diagnostic tools allows the identification of different heterogeneity determinants (Figure 2), including baseline allele-specific imbalance and post-treatment resistance mechanisms in those tumors harboring driver gene alterations. Deeper knowledge of tumor clonal evolution according to treatments will lead to more specific drug development and clinical application of personalized anticancer treatments.

8. Expert opinion

The scenario of treatment options for lung cancer patients is continuously increasing, due to acquired knowledge on comprehensive tumor molecular profiling and improvements in drug development. The possibility to obtain molecular data from NGS platforms, both from tumor tissues and blood samples, paves the way to a more profound understanding of tumor clonal evolution from diagnosis to the occurrence of treatment resistance. More in depth, the possibility to define subpopulations within apparently homogeneous patients'

categories (i.e. driver-mutant NSCLCs or PD-L1 high expressing NSCLCs, or even SCLCs), that may have different prognosis and response to treatments, represents the major expectation we raise on investigating tumor heterogeneity. Baseline co-occurring molecular alterations might predict worse outcomes and reflect the need of combinatorial strategies to delay the occurrence of treatment resistance. On the other hand, deep investigation of tumor molecular profile at the moment of acquired resistance could help in better selecting sequential treatments. Indeed, clinical and translational research is highly committed to this topic, and further results are awaited in the next years that might have significant impact on treatment selection in lung cancer patients. However, current applicability of this knowledge in the clinical practice is very limited due to several reasons. First, despite increasing recommendations and targeted NGS adoption in many countries around the globe as a firm component in the diagnostic workflow especially in diagnostic centers with high throughput, novel diagnostic tools including NGS profiling are not standard of care applied to all lung cancer patients, even within the same country. In addition, different NGS platforms are available and a clear standardization to consolidate results is lacking to date, and object of future investigations. The need of careful validation is fundamental for all different molecular assays, including NGS and RT – qPCR-based approach, before clinical implementation. Second, limited on-label use of selective TKIs and/or specific combinatorial approaches are available, outside clinical trials. Moreover, toxicity concerns arise from such personalized approach in terms of potentially increased drug-related adverse events deriving from combination strategies. Financial costs are an unsolved issue, as well, related to the extended use of comprehensive genomic profile at each step of treatment decision, but also associated to potentially exponential increase in drug development and subsequent clinical application of novel treatments. Also, the duration of treatments will impact on economic costs: very differently from chemotherapy approach, novel treatments such as TKIs and immunotherapy are commonly long-term treatments, used in a temporally continuous sequential manner, therefore determining great threat for costs sustainability for health systems. In this view, a strong commitment is eagerly required from all the stakeholders, including the researchers, the clinicians, the industries, and the governments, to converge resources in translational clinical trials, with the primary aim to standardize knowledge on tumor heterogeneity and identify the best cost-effective approach to improve treatment outcomes, and finally survival, in lung cancer patients.

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