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Opinion

Clonal Heterogeneity in Non-Small Cell Lung Cancer and the Possible Role in Predicting Response to Treatment with Immune Checkpoint Inhibitors

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Abstract

Immune oncology treatment with immune checkpoint inhibitors (ICIs) is revolutionizing therapeutic approach for advanced non-small cell lung cancer (NSCLC) patients, in terms of longer survival and improved quality of life. To date, the widely used and approved biomarker is programmed death ligand 1 (PD-L1) expression on tumour cells, but it is considered not accurate and it is more likely that many factors, related to both cancer and host, may better predict response to ICI. Among those factors, great attention is being dedicated to tumour mutation burden, defined as the number of somatic non-synonymous mutations in cancer cells assessed with next-generation sequencing technologies. Moreover, recent evidences have shown that not only the quantity of tumour mutations, but also their quality may influence response to treatment with ICI. In fact, it seems that clonal heterogeneity of cancer cells, and of predicted neo-antigens, may affect anti-cancer response in patients receiving ICI. Our aim would be to report and discuss the available evidences on this topic along with the techniques used to assess clonal heterogeneity as a

biomarker that in future might help physicians to improve NSCLC patients' selection for immune-oncology treatments.

Keywords
Non-small cell lung cancer; immune check-point inhibitors; biomarker; clonal heterogeneity; tumor mutation burden

1. Introduction

Immune oncology treatment with immune checkpoint inhibitors (ICIs) is revolutionizing management of advanced non-small cell lung cancer (NSCLC) patients, a disease which still represents the leading cause of cancer mortality in both sexes [1].

Immune checkpoints like cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) play a major role in T-cell activation and apoptosis, as well as in the maintenance of peripheral immune tolerance [2]. ICIs represent a novel class of anti-cancer agents that block these inhibitory T-cell receptors and consequentially, restore the immune system functionality in exerting an effective anti-tumor response.

In non-oncogene addicted IV stage NSCLC, monoclonal antibodies directed against PD-1, nivolumab and pembrolizumab, or against programmed death ligand-1 (PD-L1), atezolizumab, have received accelerated approval for both first and further lines of therapy [3]. Whether nivolumab and atezolizumab can be given as second or further line of systemic treatment, irrespective of PD-L1 expression on tumour cells, pembrolizumab can be administered as first or further line of therapy, whenever PD-L1 expression is at least 50% or above 1%, respectively. All of the mentioned drugs have been approved only in monotherapy, however, based on the preliminary results from the phase III trial CheckMate 227, combination of nivolumab with the anti-CTLA4 agent ipilimumab has been included in the latest ESMO guidelines as an optional regimen [3]. Similarly, even though not yet formally approved by the European Medical Agency, ESMO guidelines recommend chemotherapy combined with pembrolizumab or atezolizumab as first line of treatment in patients with PD-L1 expression below 50%.

2. Biomarkers of Response to ICI in Clinical Practice

Despite ICIs clear success, benefit is not universal and the identification of both blood-based and tissue-based biomarkers is under investigation and constitutes a major research priority. As a matter of fact, PD-L1 expression, assessed with immunohistochemisty (IHC) on tumour specimens, is the only biomarker of response in clinical practice and presents several limitations. Besides the lack of standardization in PD-L1 IHC reading, evidences have shown that PD-L1 expression seems to be a weak biomarker of response to ICIs, especially in cases of low expression. Across all tumour types, response rates of 0-17% have been reported in patients with PD-L1-negative tumours receiving ICIs, whereas in PD-L1-positive tumours, response rates range from 36% to 100% [3, 4].

To better investigate the mechanisms of response to ICIs, research has invested on alternative biomarkers, including those involved in neoantigen (neoAg) load. The term neoAg designate an immunogenic "non-self" peptide derived from tumour-specific DNA alterations, in contrast to

tumour antigens formed by non-mutated proteins with incomplete T self-tolerance due, in most cases, to restricted tissue expression pattern [5]. On a theoretical ground, neoAg load may predict response to ICI for the ability to chronically stimulate immune system, thus inducing T-cell exhaustion and PD-1 expression [6]. Therefore, also through innovative bioinformatics tools like next generation sequencing (NGS) technology, tumour mutational burden (TMB) rapidly entered the scenes of translational research. TMB, intended as the number of non-synonymous mutations within cancer genome, showed promising results in predicting response to ICI in early clinical data.

Based on the preliminary results from Checkmate 227, the first phase III clinical trial to assess prospectively the role of TMB as a predictive biomarker, a combination of nivolumab plus ipilimumab has been recommended in patients with high TMB (> 10 mutations per megabase), by the ESMO guidelines, regardless of PD-L1 expression [3, 7]. However, this early recommendation should be read with caution, considering that the final analyses of this trial are still ongoing and, so far, the only available data refer to progression free survival rate in two of the three cohorts of the study [7]. In fact, beyond the enthusiasm arisen from clinical trials, many issues are still debated. A practical concern regards the most appropriate NGS platform to assess TMB and, as a consequence, the lack of a validated threshold. The first studies evidencing an association between high TMB and response to ICIs used whole-exome sequencing (WES) on tumour specimens [8, 9]. However, despite its proven utility, WES implies high costs and expertise, making it unrealistic to be used in routine clinical practice. Therefore, numerous targeted sequencing panels based on hybrid capture-based NGS have been developed and evaluated on both tumour tissue and blood retrospectively, suggesting that TMB may be a valuable tool in predicting ICI response [10, 11]. In this respect, it is worth to mention that the possibility to assess TMB on blood seems particularly advantageous: being a non-invasive technique, it allows longitudinal assessments to monitor tumour evolution along treatment, potentially allowing to gain a deeper insight into tumour heterogeneity.

3. Neoantigen Prediction Models and Definition of Clonal Heterogeneity

From a biological perspective, the main obstacle in considering TMB as a reliable predictive tool lies in its intrinsic “quantitative” nature, since basically it represents a surrogate for neoAg load. TMB does not take into account all the passages that go from a cancer gene mutation to the development of an immunogenic neoAg. Many variables may interfere with the induction of a viable T cell response: these variables are related to the type of neoAg produced, to the way the neoAg is processed and presented on the surface on the tumour cell and to the probability that it is bounded to a Human Leucocytes Antigen (HLA) class I molecule, which is an essential step for neoAg recognition by CD8 T cells. [5, 12].

Over the past decade, neoAg prediction models have been carried out using the now available bioinformatics predictive algorithms. These methods first apply NGS to identify expressed and coding mutations within tumour, then select candidate-mutated peptides that may derive from those coding mutations and finally predict which peptides more likely may be presented by HLA class I molecules [12]. To elicit an effective anti-cancer immune response, a further step beyond neoAg presentation is neoAg ability to be recognized as foreign (immunogenicity). Immunogenicity of an Ag depends on several factors, like the type of cancer cell mutation (non-synonymous single nucleotide variant vs indel variants) and the degree the protein product differs from the wild-type

counterpart, as well as the position of the mutated amino acid within the peptide. [13, 14]. Moreover, recent evidences have shown that also neoAg heterogeneity, intended as cancer mutation clonality, plays a role in inducing T-cell response in NSCLC patients, either spontaneously or after ICI administration. As a premise, the phenomenon of tumour heterogeneity and clonal evolution in NSCLC has been deeply investigated in 2 recent works based on multi-region sequencing of surgically resected lung cancers [15, 16]. These works defined three types of genetic alterations in lung tumours: "trunk" mutations, clonal, thus present in all regions of the tumour; "branches" mutations, heterogeneous, present only in some regions of the tumour and "private" mutations, present only in one tumour region. Under an evolutionary point of view, these different types of mutations reflect the temporal development of the tumour, with trunk mutations occurring at earlier stages than branch and private mutations.

4. Clonal Heterogeneity as a Biomarker of Response to Immune Check-Point Inhibitors (ICIs)

The impact of tumour heterogeneity on the prognosis of NSCLC patients has been first outlined by the study from De Bruin et al., reporting that patients with larger subpopulations of subclonal mutations in primary tumours relapsed earlier than those with a lower extent of subclonal disease [15]. The connection between clonal heterogeneity, cancer evasion from immune system and response to ICIs, has been extensively studied by the group of McGranahan. The first work addressing this issue analysed neoAgs expression in melanoma and lung cancer samples and monitored the clonal evolution of the tumour, observing that tumours with clonal expression of neoAgs showed an increased probability to respond to checkpoint blockade [17]. More in detail, in NSCLC patients, neoAg heterogeneity was studied in two types of samples, examined alternatively with multi-region or single sample sequencing. In both cases, the specimens more often pertained to early-stage disease. Multi-region sequence analysis was available for 7 primary cultures. In this case, each coding mutation detected was classified as clonal if ubiquitously present in every tumour region sequenced within the tumour. Conversely, any mutation present only in a fraction of the tumour regions sequenced was classified as subclonal. NeoAg and clonality analysis were then performed also on a larger cohort of NSCLC patients from The Cancer Genome Atlas dataset subjected to single sample sequencing. Here, clonal status was determined assessing the cancer cell fraction for each neoAg, intended as the proportion of cancer cells harbouring the corresponding mutation.

Interestingly, in lung adenocarcinomas, neoAg load was found to be positively associated with tumour homogeneity and overall survival, independently from disease stage. This data was not confirmed in squamous cell carcinoma, despite a comparable number of predicted neoAgs. Thus, to shed light on this disparity, immune-regulatory genes expression was compared in the two histologies, evidencing a significant down-regulation of HLA molecules in the squamous cancer type. Other relevant finding was that, within the lung adenocarcinoma histotype, expression of PD-L1 and of the proinflammatory cytokine interleukin-6 (IL-6) was significantly higher in the homogeneous and high clonal neoAg group when compared to the more heterogeneous group. All the data strongly suggest that high clonal neoAg load is able to trigger a stronger T-cell response, as prompted by the presence of an inflamed microenvironment phenotype. As for response to ICI treatment, authors analyzed the clonal architecture of 34 NSCLC patients receiving pembrolizumab, and of those where WES data was available. Also in this case, both low tumour

heterogeneity and high mutational/neoAg load were positively related to PD-1 blockade efficacy with synergistic effect.

Starting from the findings of this work, the same group carried out a study focused on HLA class I expression on NSCLC cells [18]. As mentioned above, HLA functionality in the presentation of tumour neoAg plays a key role in T-cell recognition and represents the main counterpart of TMB. Bioinformatics prediction of HLA class I status may enrich TMB role as a biomarker of response to ICI treatment. Using a computational tool permitting allele-specific copy number estimation of the HLA locus, called LOHHLA (loss of heterozygosity in human leukocyte antigen), the study evidenced that loss of heterozygosity (LOH) of the HLA locus occurs in 40% of early stage NSCLC. Moreover, HLA LOH, also reflecting in down-regulation, is associated with high subclonal neoAg burden and has been observed more often in metastatic sites rather than in primary tumours. These data suggest that HLA LOH is a mechanism of immune escape occurring at more advanced stages of tumour evolution after strong selection pressures. To corroborate this hypothesis, in both lung adenocarcinomas and lung squamous cell carcinomas, subclonal cells with HLA LOH were shown to have a significantly elevated non-synonymous mutation/neoAg burden and high level of PD-L1, indicating a pre-existing immunity. Therefore, in cells with immunogenic neoAgs, loss of HLA alleles may represent a late event of immune escape, contributing to subclonal expansions and treatment resistance.

Looking at clonal heterogeneity in cancer from a different perspective, Jia et al. focused on spatial intra-tumour immune heterogeneity, in a recent study. They define immune heterogeneity as an infiltrate composed of both tumour-promoting and tumour-suppressing immune cells. In this study, surgically removed biopsies of 15 NSCLC patients were deeply analyzed at multiple sites. Among other findings, heterogeneity in the immune cell infiltration was shown to correlate with intra-tumour mutation heterogeneity.This analysis was carried out following the findings on TMB, wherein TMB correlated with clonal expansion of T-cell but not with their cytotoxic activity [19]. From an evolutionary point of view, these data may suggest that high mutation heterogeneity and its accompanying mixed immune infiltrate (which may reflect a more exhausted microenvironment) may be events occurring at later stages of tumour development as a result of immune-editing, where cancer and immune cells are involved in a mutual and continuous reshape process.

5. Future Perspectives

In a future perspective, a further understanding of the onset of subclonal events, as well as of the selection pressure that drives it, would help to find smart strategies to manipulate it. The data discussed above, prompting for clonal heterogeneity to be a later event, may justify starting ICI therapy at earlier stages of the disease. However, future longitudinal studies to confirm these hypotheses are largely needed.

To conclude, tumour clonal heterogeneity may represent one of the phenomena driving immune evasion in cancer. Thanks to the new technological and bioinformatics tools, in a near future, we expect to integrate biological data deriving from both the tumour and the host, with the final aims to better guide the selection of NSCLC patients who most likely will benefit from ICI treatment and to design tailored treatment approaches able to delay or prevent subclonal events, thus enhancing the anti-tumor effect of immune-oncology treatments.

Author Contributions

The authors contributed equally to this work.

Competing Interests

Silvia Novello declares to be Speaker Bureau and/or Advisor for BMS, Eli Lilly, BI, Astra Zeneca, MSD, Takeda, Roche. Annapaola Mariniello received financial support for travel and accommodation to participate at scientific events by Roche and BMS.

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