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Immune related endonucleases and GTPases are not associated with tumor response in patients with advanced non-small cell lung cancer treated with checkpoint inhibitors

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ABSTRACT

Immune related endonucleases have recently been described as potential therapeutic targets and predictors of response to treatment with immune checkpoint inhibitors (ICI). The aim is to evaluate the association between the expression of 5 biomarkers involved in the immune response (CD73, CD39, VISTA, Arl4d and Cytohesin-3) in parallel with the more common ICI-predictive markers, PD-L1 expression and Tumor Mutation Burden (TMB) with response to ICI therapy in an advanced non-small cell lung cancer (NSCLC) cohort.

Methods: Patients with advanced NSCLC treated with ICI single agent were divided into responders and non-responders according to RECIST v1.1 and duration of response (DOR) criteria. Immunohistochemistry was performed on pretreatment tumor tissue samples for PD-L1, CD73, CD39, VISTA, Arl4d, and Cytohesin-3 expression. TMB was estimated with NEOplus v2 RUO (NEO New Oncology GmbH) hybrid capture next generation sequencing assay. Resistance mutations in *STK11/KEAP1* and positive predictive mutations in *ARID1A/POLE* were also evaluated.

Results: Included were 56 patients who were treated with ICI single agent. The median progression-free and overall survival for the whole cohort was 3.0 (95% CI, 2.4–3.6) and 15 (95% CI, 9.7–20.2) months, respectively. The distribution of CD73 in tumor cells and CD39, VISTA, Arl4d and Cytohesin-3 expression in immune cells were not different between responders and non-responders. Also, PD-L1 and TMB were not predictive for response. The frequency of *STK11*, *KEAP1* and *ARID1A* mutations was low and only observed in the non-responder group.

Conclusion: Separate and combined expression of 5 biomarkers involved in the immune response (CD73, CD39, VISTA, Arl4d, and Cytohesin-3) was not associated with response in our cohort of advanced NSCLC patients receiving single agent ICI. To confirm our findings the analysis of independent larger cohorts is warranted.

Abbreviations: ICI, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; TMB, Tumor Mutation Burden; DOR, duration of response; VISTA, V-domain IG suppressor of T cell activation; NK, natural killer cells; IFN- γ, interferon- γ; APC, antigen presenting cells; TIL, tumor infiltrating lympocytes; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ECOG, Eastern Cooperative Oncology Group; HC, Hybrid Capture; FFPE, formalin-fixed paraffin-embedded; ExAc, Exome aggregation Consortium; dbSNP, single nucleotide polymorphism database.

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1. Introduction

Immunotherapy has revolutionized non-small cell lung cancer treatment. Presently, PD-L1 expression is the only FDA- and EMA-approved biomarker used in routine diagnostics for the stratification of immune checkpoint inhibitor (ICI) therapies. Tumor mutation burden (TMB) is approved by FDA for the treatment of adult and pediatric patients with unresectable or metastatic tumor mutational burden-high (TMB-H) [≥ 10 mutations/megabase (mut/Mb)] solid tumors, that have progressed following prior treatment and who have no satisfactory alternative treatment options [1]. Tumor cell PD-L1 expression is heterogeneously expressed and high expression (TPS ≥50%) is associated with improved tumor response to ICI [2,3]. However, some patients with low or non-expression (TPS <1%) still may respond to ICI, the predictive value is therefore low.

Endonucleases such as CD73, CD39 and VISTA (V-domain IG suppressor of T cell activation) have been suggested as potential new immunotherapeutic targets [4–7]. CD73 (for ecto-5'-nucleotidase) was suggested to be a potential biomarker of response for anti-PD-1 therapy [7]. CD73-positive natural killer cells (NK) suppress CD4-positive T cell proliferation and interferon-γ (IFN- γ) production leading to immune suppression [8]. CD39 is expressed by B cells, regulatory T cells and activated CD4 and CD8 T cells, which results in the local production of adenosine, leading to an immunosuppressive environment that promotes the progression of cancer [9,10]. VISTA is a B7 family checkpoint regulator present on hematopoietic cells, myeloid antigen presenting cells (APC), highly expressed in the tumor microenvironment that suppresses T cell activation and induces FoxP3 expression [11]. VISTA expression is associated with increased tumor infiltrating lymphocytes (TIL), PD-1 axis markers and outcome, therefore it is considered as potential therapeutic target and predictor for ICI response [6,12]. Arl4d is a GTPase whose expression is induced by the presence of PD-L1 in T cells [13]. Cytohesin-3 is a protein that can be recruited by Arl4d [14,15].

TMB is defined by the total number of mutations present in a tumor specimen [16,17]. Initially, TMB was evaluated by whole genome or whole exome sequencing [16]. As this is not applicable in the routine setting, TMB assays that rely on the genomic analysis of about 1 Mb of the coding genome have been utilized for diagnostic work [18]. In recent years, TMB has been described as a new positive predictive biomarker for immunotherapy. High TMB values showed a better response to ICI therapy independently from PD-L1 expression [16,19,20]. However, other studies found no association between TMB and tumor response, indicating that this marker is yet not useful for clinical practice [21,22]. As none of the presently available biomarkers can sufficiently discriminate responders from non-responders to ICI, we studied the role of endonucleases and GTPases for their discriminative value in advanced NSCLC. We performed a retrospective study on pretreatment tumor tissue biopsies from 56 patients with advanced NSCLC treated with mono-ICI. These tumor biopsies were evaluated for PD-L1, CD73, CD39, VISTA, Arl4d and Cytohesin-3 expression by immunohistochemistry, TMB and common clinically relevant mutations including those associated with outcome to ICI such as *STK11*, *KEAP1*, *ARID1A*, *POLE* using the targeted NEOplus hybrid capture assay [23–26].

2. Material and methods

2.1. Selection of patients and study design

We evaluated patients with advanced NSCLC from the Pius Hospital in Oldenburg and the Asklepios Klinikum Hamburg, Germany treated with Nivolumab or Pembrolizumab between 2017 and 2020. Subsequently, we selected durable responders defined as complete response (CR) or partial response (PR) or stable disease (SD) for at least 6 months versus non-responders defined as progressive disease (PD), or stable disease (SD) less than 6 months. Patients were evaluated with routine diagnostics CT for tumor response at baseline and every 6–8 weeks. Tumor response evaluation was according to the RECIST 1.1 criteria. Formalin-fixed paraffin-embedded (FFPE) material with more than 20% neoplastic cells available was selected for biomarker testing. Most biopsies came from primary tumor.

Data collected were sex, age at diagnosis, Eastern Cooperative Oncology Group (ECOG) at first immunotherapy administration, smoking status (never-smoker, current smoker, ex-smoker), number of pack years, histology, tumor response to immunotherapy, progression-free survival, and overall survival.

2.2. Biomarker tests

Both hybrid capture (HC) assay and immunohistochemistry (IHC) were performed at the Institut für Hämatopathologie Hamburg, Germany. PD-L1 was stained immunohistochemically using the antibody clone SP263 (Dako Omnis, 1:30 dilution) on the automated VENTANA BenchMark ULTRA platform (Roche Diagnostics) with positive controls of the spleen, tonsil, and placenta as part of a multi-tissue control. Scoring was conducted by board-certified and trained pathologist. The cut-off used for PD-L1 expression was as follow: in tumor cells TC0 (<1%), TC1 (1–49%) and TC2 (≥50%) and in immune cells IC0 (<1%), IC1 (1–49%) and IC2 (≥50%) from the same stained sample.

Immunohistochemistry for CD73 (ab91086, Abcam) was performed as reported previously [27]. Immunohistochemistry for the other markers was performed using the following antibodies: VISTA (D1L2G, Cell Signaling Technology), CD39 (LS-B13080, LSBio), Arl4d (LS-C369269, LSBio), Cytohesin-3 (HPA013979, Sigma Aldrich).

The expression of CD73 in tumor cells and of CD39, VISTA, Arl4d and Cytohesin-3 in immune cells were assessed by an experienced pathologist and classified as no staining (TC or IC 0) or heavy staining (TC or IC 1) (Fig. 1).

For molecular analysis, we used a commercial targeted NEOplus v2 RUO (NEO New Oncology GmbH) which detects 340 genes with their variants. Included are KRAS, *STK11*, *KEAP1*, *ARID1A*, *POLE* and it estimates TMB in an exonic territory of > 1.1 Mb [28]. At minimum three 10 μm FFPE sections were prepared and tumor tissue was micro-dissected when the tumor content was below 10%. DNA was extracted semi-automated (Maxwell®16, Promega), and 400 ng of input DNA was sonographically sheared (Covaris®) into approximately 200 bp double-stranded fragments.

Hereafter, adapters were ligated, and genomic regions of interest were enriched using complementary bait sequences. In this hybrid capture, the selected baits ensure optimal coverage of all relevant genomic regions, in a 1.14 Mb complete genomic territory size. Next, clonal amplification and sequencing of the targeted fragments was performed with next generation sequencing (NextSeq 500/550, Illumina). The mutation identification was performed using NEO New Oncology's proprietary computational biology analysis pipeline and the analysis performed using NEO diagnosis software. TMB analysis was performed as recently reported [27]. In short, the number of somatic mutations were quantified and extrapolated to the whole exome using a validated algorithm (NEO New Oncology, GmbH). Alterations known in Exome Aggregation Consortium (ExAc) or Single Nucleotide Polymorphism Database (dbSNP) were excluded. Variants with an allelic frequency of at least 10% (for LOD 0.1) were included. The TMB value was provided as mutations per Megabase (mut/Mb) with an updated 2020 NEO algorithm. A cut-off of 10 mutations per Mb was used for high versus low TMB.

2.3. Statistics

Descriptive statistics are given for patient and tumor characteristics. Chi-square test was used to compare IHC staining results in responders and non-responders. Spearmann Rank test was used to evaluate combinations of markers. The progression-free survival (PFS) is the time between the first day of receiving immune checkpoint inhibitor until

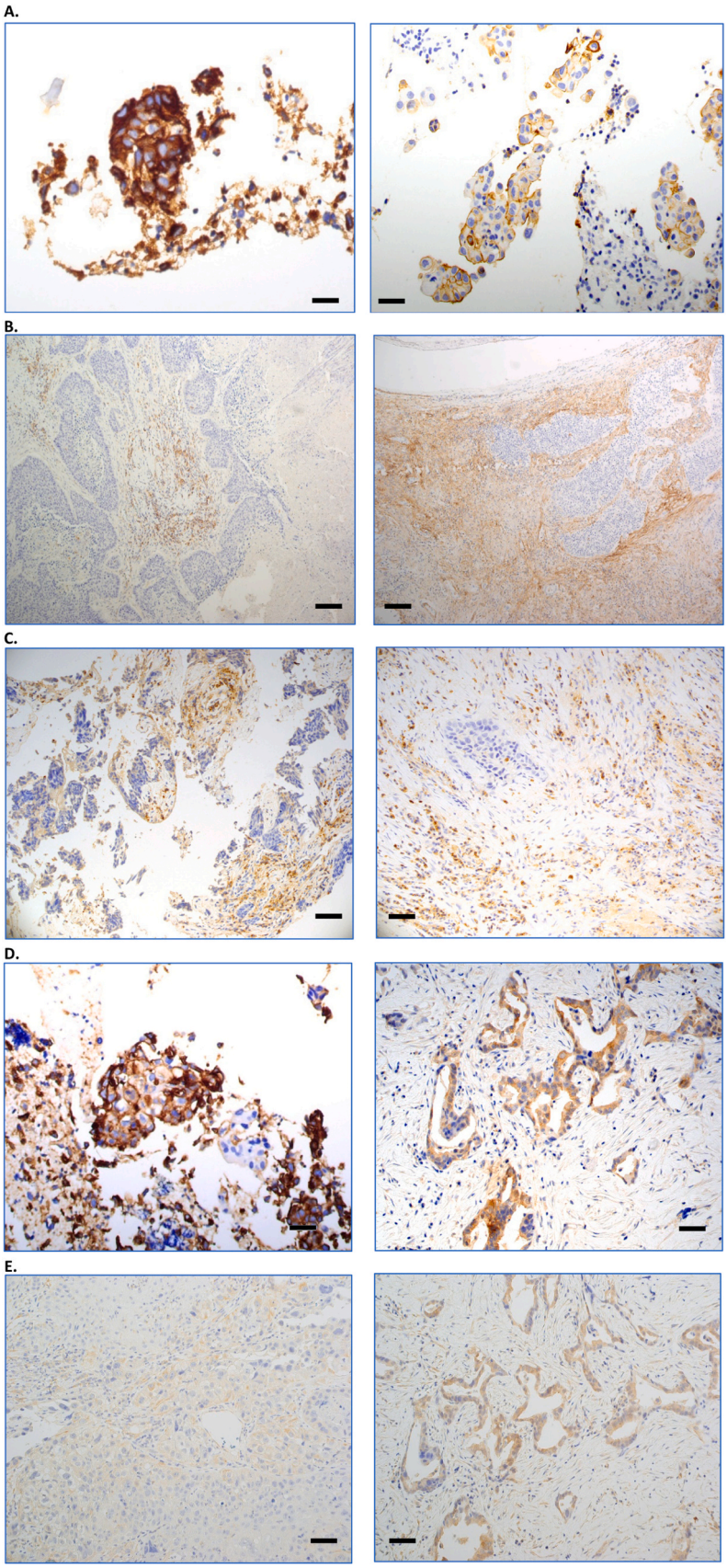

Fig. 1. IHC staining for CD73 in tumor cells TC1 (A) [scale bar 100 μm], CD39 in immune cells IC1 (B) [scale bar 1000 μm], VISTA in immune cells IC1 (C) [scale bar 400 μm], Arl4d in immune cells IC1 (D) [scale bar 250 μm] and Cytohesin-3 in immune cells IC1 (E) [scale bar 250 μm].

tumor progression was observed with CT scans. The overall survival (OS) is the time between the first day of receiving ICI and death of patient. PFS and OS were estimated with Kaplan-Meier method.

3. Results

3.1. Patients

Patients were divided into two groups according to their response to therapy: 24 (42.9%) responders and 32 (57.1%) non-responders. Nivolumab was administered in 45 (80.4%) of the 56 patients and pembrolizumab in 11 (19.6%). Twenty (35.7%) patients received ICI in first line versus 36 (64.3%) in second line or further lines. Most patients enrolled in the study were male smokers. Patient and tumor characteristics are described in Table 1.

3.2. Expression of IHC markers PD-L1, CD73, VISTA, CD39, Arl4d and Cytohesin-3 in relation to clinical response

PD-L1 expression in tumor tissue was assessed in 55 patients. In the responder group, only 17% had a TC0 score and 83% had at least a TC1. In the non-responder group, 31% of patients had TC0 whereas 66% had at least TC1 (Fig. 2, supplement Table 1). The PD-L1 expression distribution in tumor cells and immune cells was not different between responders and non-responders (Chi-square 1.75; $p = 0.41$).

The CD73 expression was available for neoplastic cells in tumor tissue of 48 patients. The overall CD73 expression distribution between responders and non-responders was not different (Fig. 2) (Chi-square value 4.65, $p = 0.32$).

The VISTA expression was available for immune cells in tumor tissue of 48 patients. A numerical difference in expression was observed for IC1 score: 41% vs. 31% in responders versus non-responders, respectively (Fig. 2). However, the overall VISTA expression distribution was not different between responders and non-responders (Chi-square value 2.29, $p = 0.68$).

The CD39 expression was available for immune cells in tumor tissue of 31 patients. The CD39 score 0 and 1 appeared to be overlapping in both groups (Fig. 2). The overall CD39 distribution was not different between responders and non-responders (Chi-square 0.07, $p = 0.99$).

The Arl4d expression was available for immune cells in tumor tissue of 44 patients. A numerical difference in expression was observed in the low expressors for the non-responder group which was slightly higher in comparison with the responder group IC0, 65% versus 56%, respectively. The overall Arl4d distribution was not different between both groups (Chi-square 1.26, $p = 0.74$).

The Cytohesin-3 expression was available for immune cells in tumor tissue of 43 patients. Here, we observed a higher number of low expressors in non-responders 92% in comparison with the responder group 72% (Fig. 2). In the responder group, we observed a higher IC1 distribution with 28% in comparison with the non-responder group 8%. However, the distribution was not different between responders and non-responder group (Chi-square 3.1, $p = 0.38$).

Table 1
Patients and tumor characteristics according to tumor response groups.

Variable		Total (n = 56) (%)	Responders (n = 24) (%)	Non-responders (n = 32) (%)
Age	Median (± SD)	65 (8.2)		
	Range	45–80		
Gender	Male	41 (73.2)	19 (79.2)	22 (68.8)
	Female	15 (26.8)	5 (20.8)	10 (31.3)
ECOG PS	0	14 (25.0)		
	1	35 (62.5)		
	≥2	7 (12.5)		
Histology	Adenocarcinoma	25 (44.6)	10 (41.7)	15 (46.9)
	Squamous-cell carcinoma	28 (50.0)	13 (54.2)	15 (46.9)
	Other	3 (5.4)	1 (4.2)	2 (6.3)
Smoking status	Current smoker	39 (69.6)	17 (70.8)	22 (68.8)
	Ex-heavy smoker	13 (23.2)	7 (29.2)	6 (18.8)
	Never smoker	4 (7,1)		4 (12.5)
Median packyears	(median / range)	35 (0–90)		
Treatment	Nivolumab	45 (80.4)		
	Pembrolizumab	11 (19.6)		
Therapy Line (Checkpoint inhibitor)	First line	20 (35.7)	9 (37.5)	11 (34.4)
	Second line	26 (46.4)	11 (45.8)	15 (46.9)
	Third line or more	10 (17.9)	4 (16.6)	6 (18.8)
Tumor mutation burden (n = 32)	High TMB > 10mt/mb	18 (56.3)	7 (53.8)	11 (57.9)
	Low TMB < 10mt/mb	14 (43.8)	6 (46.2)	8 (42.1)

3.3. TMB and driver mutations

A total of 32 patients had enough tumor material and were tested for TMB using the cut-off of 10 mutations per Mb to discriminate high versus low TMB (Fig. 3). Overall, 56.3% patients had a high TMB. Among those 53.8% (7/13) in the responder group and 57.9% (11/19) in the non-responder group (Table 1).

Gene variants were mostly observed in non-responders (Fig. 4). In *TP53, KRAS* and *STK11* genes numerically more mutations were observed in the non-responders compared to the responder group (84% (16/19) versus 69% (9/13), and 37% (7/19) versus 31% (4/13), 11% (2/19) versus 0% (0/13)), respectively.

Two *STK11* mutations were observed in the non-responder group, one pathogenic (p.W308 *) and the other likely pathogenic (p.K62 *). The mutation in *KEAP1* is likely pathogenic (p.N189fs) and present in 5% (1/19) non-responders. *ARID1A* pathogenic mutation (p.Q878 *) occurred in one non-responder patient. No pathogenic mutations in *POLE* were observed in this cohort (supplementary table 2).

3.4. Combination of biomarkers

In immune cells of tumor tissue Arl4d expression was associated with Cytohesin-3 expression (Spearman Rank 0.69, $p = 0.001$). A higher TMB was associated with a lower Arl4d expression in immune cells of tumor tissue (Spearman Rank 0.38, $p = 0.04$) (Table 2).

3.5. Progression free and overall survival

The median PFS and OS for the whole cohort were 3.0 (95% CI, 2.4–3.6) and 15 (95% CI, 9.7–20.2) months, respectively. PD-L1, CD73, CD39, VISTA, Arl4d and Cytohesin-3 expressions were not associated with PFS and OS, as was high versus low TMB.

4. Discussion

In this study we first analyzed the expression of PD-L1. For the highly dynamic expression of PD-L1, studies either showed tumor responses or absence of response [2,29,30]. Our PD-L1 data showed that this biomarker alone does not differentiate between responders and non-responders. This can be explained by differences in the timing and source of collection of the samples, the heterogeneity of expression of PD-L1 in the same sample [31]. The PD-L1 expression in immune cells also did not show a difference in distribution in responders and non-responders. This is in agreement with observation that the expression in immune cells is mainly described in patient cohorts receiving atezolizumab (anti-PD-L1 therapy) [32]. In our study, the patients

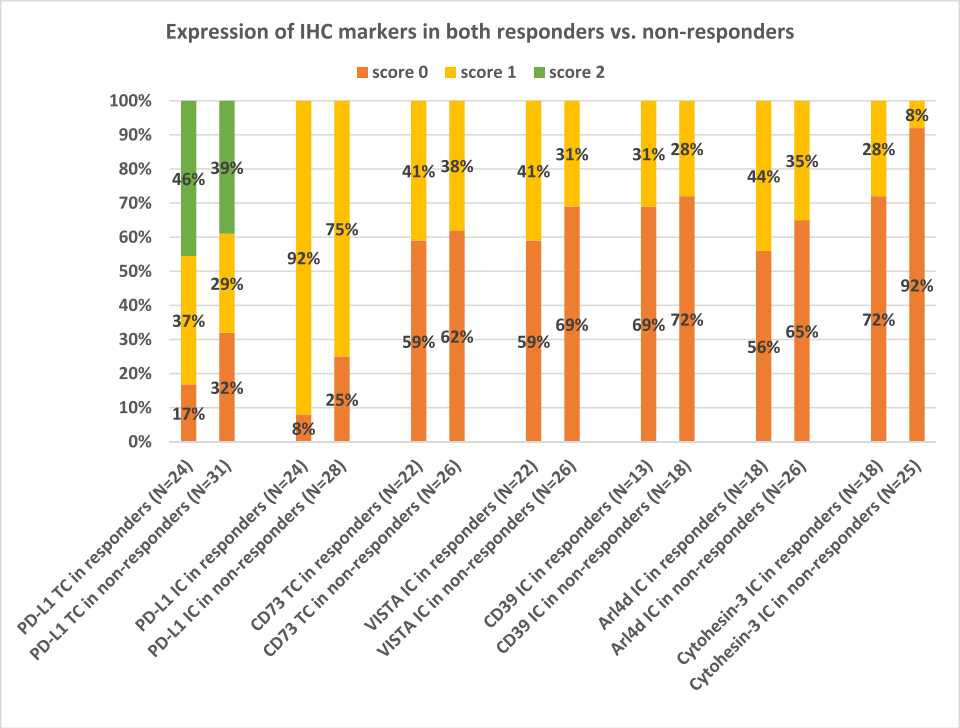

Fig. 2. Distribution of PD-L1 in tumor cells and in immune cells and the distribution of CD73 in tumor cells and VISTA, CD39, Arl4d and Cytohesin-3 in immune cells in responder group and non-responders group.

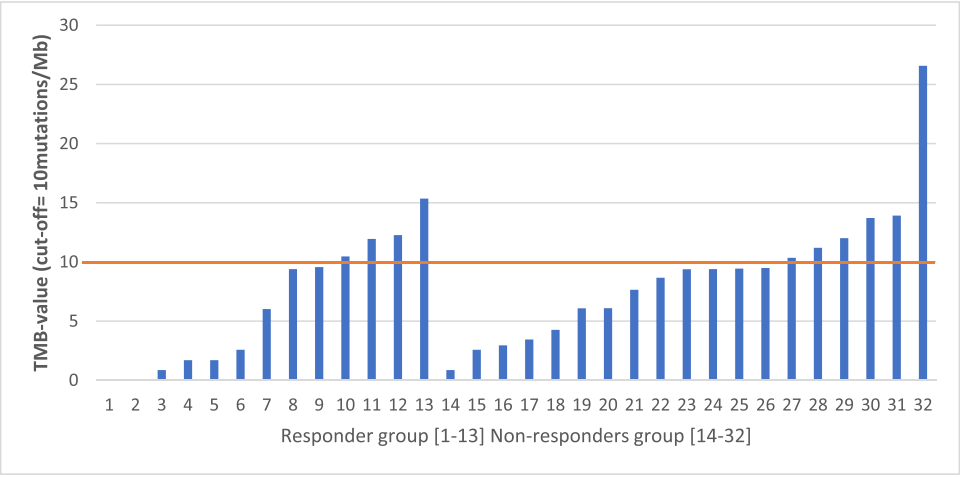

Fig. 3. Distribution of TMB in patients with advanced NSCLC treated with checkpoint inhibitors. TMB was available in 13/24 responders and 19/32 non-responders.

received either nivolumab or pembrolizumab, both anti-PD-1 therapies.

Here, tumor samples of patients with advanced NSCLC treated with ICI were used to evaluate whether differential expression of the new potential predictive biomarkers CD73 on tumor cells, CD39 on immune cells and VISTA expression on immune cells are associated with tumor response. The distribution of CD73, CD39 and VISTA expression either on tumor or immune cells was not different between responders and non-responders. CD73 expression in tumor cells was reported as being a potential biomarker for response to anti-PD-1 therapy because of its ability to suppress the immune response activated from the checkpoint blockade [7]. Elevated CD39 expression in several cancer types is associated with poor outcome [33–35]. VISTA has been demonstrated as a marker of acquired resistance to anti-PD-1 therapy in melanoma [36]. In NSCLC it is mainly described as being a potential target for immunotherapy [6,12].

For the first time, Arl4d and Cytohesin-3 expression have been investigated in NSCLC tumor tissue. Since the expression of Arl4d is induced by the presence of PD-L1 and Cytohesin-3 can be recruited by Arl4d, we hypothesized that the expression of both these proteins would be different between the responders and non-responders. In this study, we observed that there was no association between the expression of both Arl4d and Cytohesin-3 and the response to ICI. However, we found a strong correlation between the expression of Arl4d and Cytohesin-3 in immune cells from NSCLC (Spearman Rank 0.69, $p = 0.001$). Also, we observed a trend in higher Cytohesin-3 expression the responders compared to non-responders, although the numbers are small. These observations are of potential interest in the role of these markers in NSCLC. Arl4d interferes with signal transduction via PI3K/Akt axis, which leads to IL-2 inhibition and seems to be regulated via co-signaling after TCR stimulation. Cytohesin-3, who interacts strongly with

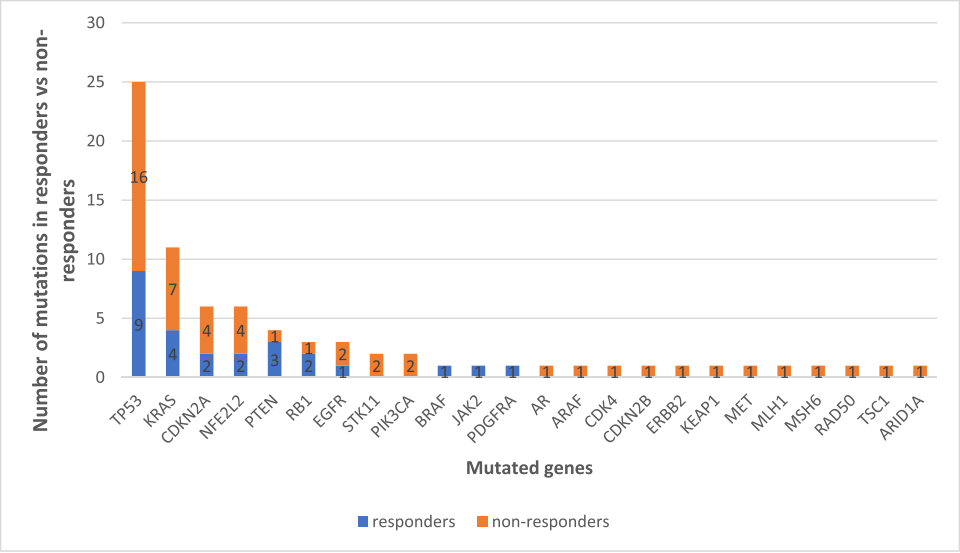

Fig. 4. Distribution of gene variants in responders and non-responders of advanced NSCLC patients on ICI.
(Blue: total number of responders n = 13 / Orange: non-responders n = 19).

Table 2
Spearman's Rank coefficient analysis of biomarker expression in responder and non-responder patients with advanced NSCLC.

	PD-L1 TC	PD-L1 IC	CD73	VISTA	CD39	Arl4d	Cytohesin-3	TMB
PD-L1 TC N	1 55							
PD-L1 IC N	0.56* 52	1 52						
CD73 N	0.09 48	0.27 46	1 48					
VISTA N	0.07 48	0.29 46	0.02 48	1 48				
CD39 N	0.01 30	0.07 29	- 0.15 28	0.11 28	1 31			
Arl4d N	0.18 43	0.06 40	0.17 39	0.10 39	- 0.16 26	1 44		
Cytohesin-3 N	0.10 42	0.29 40	0.15 38	0.22 38	- 0.20 28	0.69* 40	1 43	
TMB N	0.21 33	- 0.15 30	- 0.33 29	0.23 29	0.19 21	0.38** 29	- 0.32 28	1 33

phosphatidylinositol-3,4,5-triphosphate (PIP3), is regulated by PI3K and has been described in T-cell anergy [37]. More studies including this biomarker should be performed in order to better understand the way these molecules interact with each other and affect the immune system.

Our analysis of TMB as a potential predictive biomarker in our cohorts revealed that the proportion of high TMB was not different between responders and non-responders. The performance of the NEO New Oncology test used for TMB estimation was previously reported to correlate with the Foundation Medicine F1Dx panel and as part of the German Harmonization trial, demonstrated to compare very well with F1Dx [18,27]. However, this study was performed independently of any clinical response to ICI (or other treatments). The present study evaluated the value of the TMB assay in a cohort of patients treated with ICI as potential predictor for tumor response. Meanwhile many other TMB tests were reported and there is quite some debate on the use of different TMB cut-offs especially using different assays, differences in variant composition and the number of mutations/Mb [18]. Therefore, differences between the various TMB panels might explain why the used cut-offs are not yet optimal.

Mutations in *KEAP1* and *STK11* have been reported to be associated with resistance to ICI therapy and those in *ARID1A* were found to correlate with better outcome in patients with NSCLC receiving ICI [23–25]. In this study, too few mutations were observed to make conclusions.

The most studied combination is TMB with PDL-1 expression. This revealed that adding TMB to the standard PD-L1 expression test has a predictive value for the response to immunotherapy [38]. In our cohort, we observed that both high TMB and high PD-L1 expression did not yield a better response to ICI.

The different IHC markers were independent of each other, only Arl4d and Cytohesin-3 were associated with each other (Table 2). This study showed that a combination of IHC biomarkers did not improve the tumor response prediction in our cohort of 56 patients.

The strong point of our exploratory cohort was the inclusion of clinical endpoints such as tumor response towards ICI only. Next to TMB and the known IHC markers PD-L1, VISTA, CD39 and CD73, we used Arl4d and Cytohesin-3 expression in immune cells in NSCLC tumors.

One of the limitations in this study is the lack of sufficient tumor tissue in routine biopsies of patients with advanced NSCLC. A possible solution is the inclusion of liquid biopsies in diagnostic analysis as a source to test for predictive markers in tumor-derived circulating DNA [39]. Another limitation is the relative low number of patients, particularly for subgroup analyses. Therefore, to confirm our findings the analysis of independent larger cohorts is warranted.

Disclosures

HOR declarations of interest: none.

MF declarations of interest: none.

LCH declarations of interest: advisory role fees from Boehringer Ingelheim, Pfizer and Molecular Health.

SS declarations of interest: none.

MT declarations of interest: consulting fees from BMS, MSD, Roche, Boehringer Ingelheim, AstraZeneca; Speaker fees and advisory board from AstraZeneca, Boehringer Ingelheim, BMS, MSD, Roche Takeda.

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LD declarations of interest: none.

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CRediT authorship contribution statement

H.O. Ramdani: Formal analysis, Investigation, Visualization. **M. Falk:** Investigation, Writing – review & editing. **L.C. Heukamp:** Methodology, Supervision, Writing – review & editing. **S. Schatz:** Writing – review & editing. **M. Tiemann:** Resources. **C. Wesseler:** Resources. **L. Diehl:** Writing – review & editing. **E. Schuuring:** Methodology, Project administration, Supervision, Writing – review & eding. **H.J.M. Groen:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **F. Griesinger:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Appendix A. Supporting information

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