Original Article

Blood tumor mutational burden and dynamic changes in circulating tumor DNA predict response to pembrolizumab treatment in advanced non-small cell lung cancer

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Background: The use of immunotherapy targeting the programmed cell death protein-1 (PD-1) and its ligand (PD-L1) has provided new hope for patients with non-small cell lung cancer (NSCLC). However, good biomarkers are needed to identify which patients will benefit from the treatment. In this study, we investigated if circulating tumor DNA (ctDNA) could predict response to pembrolizumab.

Methods: Plasma samples from patients with NSCLC treated with pembrolizumab were collected immediately before and after one or two cycles of treatment. ctDNA was isolated and analyzed using targeted next-generation sequencing with a lung cancer gene panel.

Results: Mutations were detected in ctDNA in 83.93% of patients before treatment initiation. High blood tumor mutational burden (bTMB), measured as the number of different mutations per Mb panel, correlated to longer progression-free survival (PFS) (10.45 *vs.* 2.30 months) and overall survival (OS) (21.80 *vs.* 12.20 months), whereas no predictive value was found in the number of mutant molecules per mL of plasma. The absence of mutations just after treatment initiation correlated with improved PFS (20.25 *vs.* 4.18 months) and OS (28.93 *vs.* 15.33 months). High bTMB before treatment was associated with a decreasing ctDNA level after treatment initiation. Importantly, a subgroup of patients experienced an increase in the ctDNA level after treatment initiation, and this correlated with inferior PFS (2.19 *vs.* 11.21 months) and OS (7.76 *vs.* 24.20 months). All patients in the subgroup with increased ctDNA level progressed within 10 months.

Conclusions: Monitoring of ctDNA contains vital information about response to therapy, where the bTMB and the dynamics in the initial part of treatment are particularly important for response. Increasing ctDNA levels after treatment initiation are significantly correlated with inferior survival.

Keywords: Immunotherapy; lung cancer; biomarkers; circulating tumor DNA; liquid biopsy

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Introduction

Lung cancer is one of the leading cancers worldwide, both in terms of incidence and mortality (1). Non-small cell lung cancer (NSCLC) constitutes the majority of lung cancer cases, and these patients often present with advanced disease at the time of diagnosis (2). The use of immunotherapy targeting the programmed cell death protein-1 (PD-1) and its ligand (PD-L1), such as the anti-PD-1 drug pembrolizumab, has improved progression-free survival (PFS) and overall survival (OS) and has become a part of the standard-of-care for patients with NSCLC (3-5). The KEYNOTE-024 trial tested the efficacy of pembrolizumab versus platinum-based chemotherapy in a first-line setting for NSCLC with a PD-L1 tumor proportion score (TPS) of at least 50%. The study showed that treatment with pembrolizumab correlated with longer PFS and OS with fewer treatment-related adverse events. Furthermore, response rate and duration of response were superior in the pembrolizumab-treated group (3,5). Subsequently, the KEYNOTE-042 trial was commenced to investigate if pembrolizumab treatment remained superior to platinum-based chemotherapy when the PD-L1 TPS threshold was lowered to 1%. The study showed that pembrolizumab treatment prolonged OS significantly, however, the greatest efficacy was observed in the patient subgroup with a PD-L1 TPS of at least 50%. Surprisingly, the study found no significant difference in PFS (4).

At the time of inclusion for this study, the expression level of PD-L1 was used to determine eligibility for treatment with pembrolizumab, with patients with a PD-L1 TPS of at least 50% qualifying for first-line treatment, and patients with a PD-L1 TPS of at least 1% qualifying for second-line treatment (6). However, the response to treatment differs vastly between patients and good biomarkers, besides PD-L1 expression, are needed to identify which patients will benefit from pembrolizumab treatment (3-5). Previously, studies have found that the mutational status of *KRAS* and *TP53* are predictive of a longer PFS and OS, while mutations in the *ERBB*-family genes and *ALK* predict inferior survival (7-9). Studies have found that a high tumor mutational burden (TMB), a measure of non-synonymous somatic mutations per Mb identifies patients who respond to immunotherapy, which can be estimated from either tissue or liquid biopsies (10-12). This measure reflects the quantity of detected mutations and the coverage of the employed panel in targeted sequencing.

Most of the earlier studies have been conducted on tumor tissue, which can be challenging to obtain, can be a hazardous procedure for the patient, and may fail to capture the spatial heterogeneity and complexity of the cancer (13,14). The use of liquid biopsies in the form of blood samples containing cell-free DNA (cfDNA), which includes circulating tumor DNA (ctDNA), offers new potential for cancer patients, both in the form of assessment of tumor burden, detection of response or resistance to treatment and relapse (13,15,16). Furthermore, ctDNA offers the possibility of longitudinal monitoring during treatment of cancer (17). In this study, the analysis of ctDNA from patients with NSCLC treated with pembrolizumab will be used to identify the patient subgroup(s) that benefits from pembrolizumab treatment. We wished to investigate if liquid biopsies obtained immediately before and after one or two cycles of treatment will contain predictive factors for treatment response using either the blood tumor mutational burden (bTMB) or the average number of mutant molecules per mL of plasma (aMM). We present this article in accordance with the REMARK reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-818/rc).

Highlight box

Key findings

- The baseline blood tumor mutational burden and the dynamics in ctDNA during the initial weeks of treatment are associated with response to pembrolizumab.

What is known and what is new?

- Former studies have shown that the tumor mutational burden and clearing of ctDNA predicts response to immune checkpoint inhibitors.
- This manuscript shows that the blood tumor mutational burden, but not the level of ctDNA before treatment initiation is important for response. Furthermore, we show that increasing levels of ctDNA during treatment is important in identifying patients with little to no benefit from treatment.

What is the implication, and what should change now?

- ctDNA could be used as a monitoring tool during the initial part of treatment which will distinguish responders from non-responders.

Methods

Patients

Patients with advanced NSCLC treated with pembrolizumab as first- or second-line palliative treatment at Aarhus University Hospital, Denmark in the period

between January 2017 and April 2019 were evaluated for enrollment in this retrospective study. The patients received an intravenous infusion of 2 mg/kg of pembrolizumab every three weeks. Patients who died within a month from treatment start were excluded. A total of 56 patients had available plasma samples taken immediately before pembrolizumab initiation (T_0) and were enrolled in the study. Forty-one patients had an additional plasma sample available after one or two cycles of treatment (T_x). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and all patients provided written informed consent. The study was approved by the regional ethics committee of Region Midt (Approval No. 1-16-02-211-16).

Sample collection and preparation

Peripheral blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes immediately before the first dose of pembrolizumab and every three weeks afterward. The blood samples were centrifuged at 1,400 g for fifteen minutes, and the plasma and buffy coats were isolated and frozen in separate aliquots at −80 ℃. Plasma samples taken before treatment initiation and after two cycles of treatment were chosen for analysis to ensure enough time for an effect of the treatment. Twenty-one patients had no sample available after two cycles of treatment, however, 6/21 patients had a sample collected after one cycle which was included instead. The cfDNA was isolated from the available plasma (2.00–7.30 mL) using AVENIO cfDNA Isolation Kit (Roche, Basel, Switzerland) and eluted in 65 µL elution buffer. DNA was isolated from 0.20 mL buffy coat using AVENIO Tumor DNA Isolation and QC Kit (Roche) and AVENIO Tumor Cleanup and Capture Beads (Roche) and eluted in 30 µL elution buffer. The quality of the isolated cfDNA and DNA was analyzed using QubitTM dsDNA HS assay kit (Thermo Fischer Scientific, Waltham, MA, USA) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Next-generation sequencing

The isolated cfDNA were prepared for sequencing using the AVENIO ctDNA Surveillance Kit (Roche) with a median cfDNA input of 32 ng (9–55 ng). The DNA from buffy coats was fragmented and polished using AVENIO Library Prep Kit (Roche) and afterward prepared using the reagents from the AVENIO ctDNA Targeted Kit (Roche) with a DNA input of 25 ng. The samples were sequenced using targeted sequencing against a 0.198 Mb panel, containing 197 lung cancer-related genes on a NextSeq 500 High Output Lane (Illumina, San Diego, CA, USA) in a multiplex of 16 samples. The data was processed using AVENIO Oncology Analysis Software v. 2.0.0 (Roche) and the build-in RocheDefault filter set, which excludes variants with an allele frequency (AF) >0.10% in Exome Aggregation Consortium (ExAC), 1000 Genomes Project or the Single Nucleotide Polymorphism database (dbSNP Common). Variants were included if they have been reported and somatically confirmed in the Catalogue of Somatic Mutations in Cancers (COSMIC), The Cancer Genome Atlas (TCGA), or the Loci of Interest list. Furthermore, synonymous variants were excluded, and non-synonymous variants were included if they presented with an AF of at least 0.10% and a minimum of three unique variant reads. To avoid inclusion of non-cancerous variants, we sequenced buffy coat DNA from patients with mutations we suspected could be single nucleotide polymorphisms or clonal hematopoiesis-derived mutations. Patients were selected for buffy coat analysis if they had an identical mutation at both timepoints (T_0 and T_x) at a similar AF (less than 50% change) (18). We furthermore sequenced buffy coat DNA from patients with mutations with an AF over 30%, which we suspect could be non-somatic. This resulted in a subpopulation of eleven patients, whose buffy coat DNA was chosen for analysis. Mutations present in both plasma and buffy coat samples were excluded. The remaining mutations per patient were summed and divided by the size of the panel (0.198 Mb) to gain the bTMB measured as mutations per Mb.

Statistical analysis

Kaplan-Meier survival curves were used to compare PFS and OS between patient subgroups, and the differences were determined with the log-rank (Mantel-Cox) test for P values and Cox regression analysis for hazard ratio (HR). PFS was defined as the time from treatment initiation to the date of first detected radiological progression, determined using the RECIST criteria, or death. If patients had not yet progressed, they were censored at the date of their last follow-up scan before the data cutoff date of December 20, 2021. Duration of treatment was calculated as the months between the first and last treatment with pembrolizumab, with patients discontinuing after a single cycle of treatment being noted as having a duration of

treatment of three weeks (0.69 months), based on the length of a pembrolizumab treatment cycle. OS was defined as the time from treatment initiation to the date of death. Patients without complete survival data were censored at the data cutoff date. The number of mutant molecules per mL of plasma was used as a measure for ctDNA level. This number was calculated for each mutation as [Isolated DNA mass (ng)]*330*(Allele Fraction)/[Plasma volume (mL)], with 330 being an estimate for the number of genome equivalents per ng of DNA. The aMM was calculated for each patient. Fisher's exact test was used to determine correlation between line of treatment and bTMB and to correlate bTMB and aMM with the best overall response, with patients without available scans being excluded from this analysis. Association between continuous variables was determined using linear regression. Differences in the bTMB and the number of mutant molecules per mL of plasma were calculated using Wilcoxon's matched-pairs signed rank test for paired samples and Mann-Whitney test for unpaired samples. Univariate Cox regression analysis was employed to evaluate the association between variables and PFS or OS. Independent variables with P values below 0.05 in the univariate analysis were included in a multivariate Cox regression analysis. All tests were two-sided, and P values below 0.05 were considered significant. The statistical analyses and visualization of data were carried out in GraphPad Prism version 9.1.1 (GraphPad Software, San Diego, CA, USA) and RStudio version 2021.09.1 (RStudio, Boston, MA, USA).

Results

Patients

Fifty-six patients with advanced NSCLC treated with pembrolizumab as first- or second-line treatment and with available baseline (T_0) plasma samples were included in the study. Forty-one/56 patients (73.21%) had an additional plasma sample after one or two cycles of treatment (T_x) available with a median time from treatment initiation to sampling of 1.41 months (range, 0.69–2.96 months).

Forty-five patients (80.36%) had progressed on pembrolizumab treatment at the time of data cutoff, five patients (8.93%) had died before progression, and one patient (1.79%) was censored in the PFS analyses at the last follow-up due to non-compliance. 44 patients (78.57%) had died at the time of data cutoff. Median PFS and OS for the patient cohort was 5.08 months (range, 0.46–51.78 months) and 19.81 months (range, 1.18–55.89 months), respectively. A summary of the duration of treatment and survival is shown in *Figure 1*.

To investigate the influence of baseline clinical characteristics on PFS and OS, Cox regression analysis was employed. The univariate analysis found no significant association between histology, sex, smoking, line of treatment, performance status, co-morbidities or PD-L1 TPS and PFS or OS. The only baseline clinical characteristics identified as statistically significant in the univariate analysis was age in correlation to PFS (HR =1.04; 95% confidence interval (CI): 1.01–1.08, P=0.03), however, age was not significant in terms of OS (HR =1.02; 95% CI: 0.98–1.06; P=0.31). Age remained an independent predictor of PFS after multivariate analysis (HR =1.07; 95% CI: 1.02–1.13; P=0.01) (Table S1).

Baseline bTMB but not the level of mutant molecules per mL is predictive of response

The ctDNA and buffy coat samples collected at T_0 were sequenced at a median unique depth of 4,030.50 (range, 1,444–9,073) for ctDNA and 3,576 (range, 1,899–7,425) for buffy coat samples. A total of fifteen variants were classified as clonal hematopoiesis-derived variants as they were identified in the paired ctDNA and buffy coat samples and were therefore excluded from further analysis (Table S2).

Sequencing of T_0 ctDNA led to the detection of mutations in 47 patients (83.93%). A total of 138 mutations in 46 different genes were detected, with a median bTMB of 10.10 mutations per Mb (range, 0–50.51 mutations per Mb) (*Figure 2A*,*2B*).

To investigate if the number of mutations at T_0 was predictive of treatment response, patients were allocated into two subgroups based on the median bTMB: low bTMB (median bTMB or below) and high bTMB (above the median bTMB). This demonstrated that a high bTMB (n=25) was associated with a significantly longer PFS with a median of 10.45 *vs.* 2.30 months for the subgroup with a low bTMB (n=31) (HR =0.49; 95% CI: 0.28–0.87; log-rank P=0.02) (*Figure 2C*). OS was also longer for the subgroup with a high bTMB with 21.80 *vs.* 12.20 months (HR =0.52; 95% CI: 0.27–0.95; log-rank P=0.03) (*Figure 2D*). Multivariate Cox regression analysis showed that high bTMB remained an independent predictor of PFS (HR =0.31; 95% CI: 0.13–0.68; P=0.005), and OS (HR =0.48; 95% CI: 0.23–0.97; P=0.05) (Table S1). Patient characteristics stratified by bTMB are shown in *Table 1*,

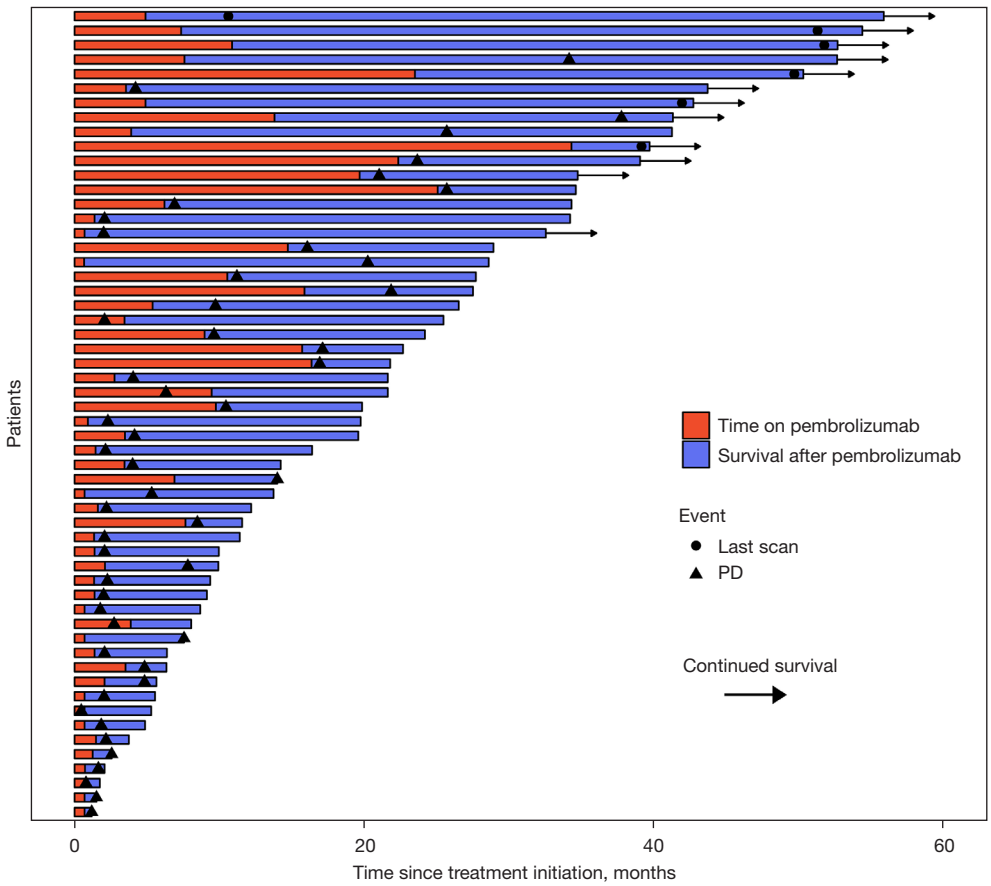

Figure 1 Swimmer plot for all included advanced NSCLC patients treated with a least one cycle of pembrolizumab. Each bar represents an individual patient. PD, progressive disease; NSCLC, non-small cell lung cancer.

where no significant difference between the high and low bTMB subgroups was found. Alongside superior PFS and OS, we also identified a significant correlation between high bTMB and favorable radiological response using Fisher's exact test (complete or partial response as the best overall response) (P=0.01) (Table S3). Using linear regression, no association was found between the bTMB and neither the volume of plasma (R^2=0.001, P=0.79) nor the level of input cfDNA (R^2=0.01, P=0.44) (Figure S1).

The level of ctDNA was measured as the aMM. The patients had a median aMM of 52.44 mutant molecules per mL of plasma (range, 0–15,600 mutant molecules per mL of plasma). We examined if aMM was likewise predictive of response by stratifying patients based on above or below median aMM (52.44 mutant molecules per mL of plasma). Interestingly, no difference was found in survival between patients with a low or high aMM neither for PFS (6.61 *vs.* 4.09 months, HR =1.30; 95% CI: 0.74–2.28; log-rank P=0.35) nor OS (23.00 *vs.* 15.33 months, HR =1.42; 95% CI: 0.78–2.59; log-rank P=0.25) (*Figure 2E,2F*).

The most frequently mutated genes were *TP53* and *KRAS*, which constituted 24.64% and 15.94% of all identified mutations, and were mutated in 30 patients (53.57%) and 21 patients (37.50%), respectively. No association was found between *TP53* and *KRAS* mutations and PFS or OS using Cox univariate analysis (Table S1). A list of genetic alterations is provided in Table S4. The association between line of treatment and bTMB and aMM was investigated using Fisher's exact test. No significant association was detected neither for bTMB (P=0.11) nor aMM (P=0.53).

ctDNA dynamics in the initial weeks of treatment predict clinical outcome

Next, we wanted to investigate if the T_x samples contained

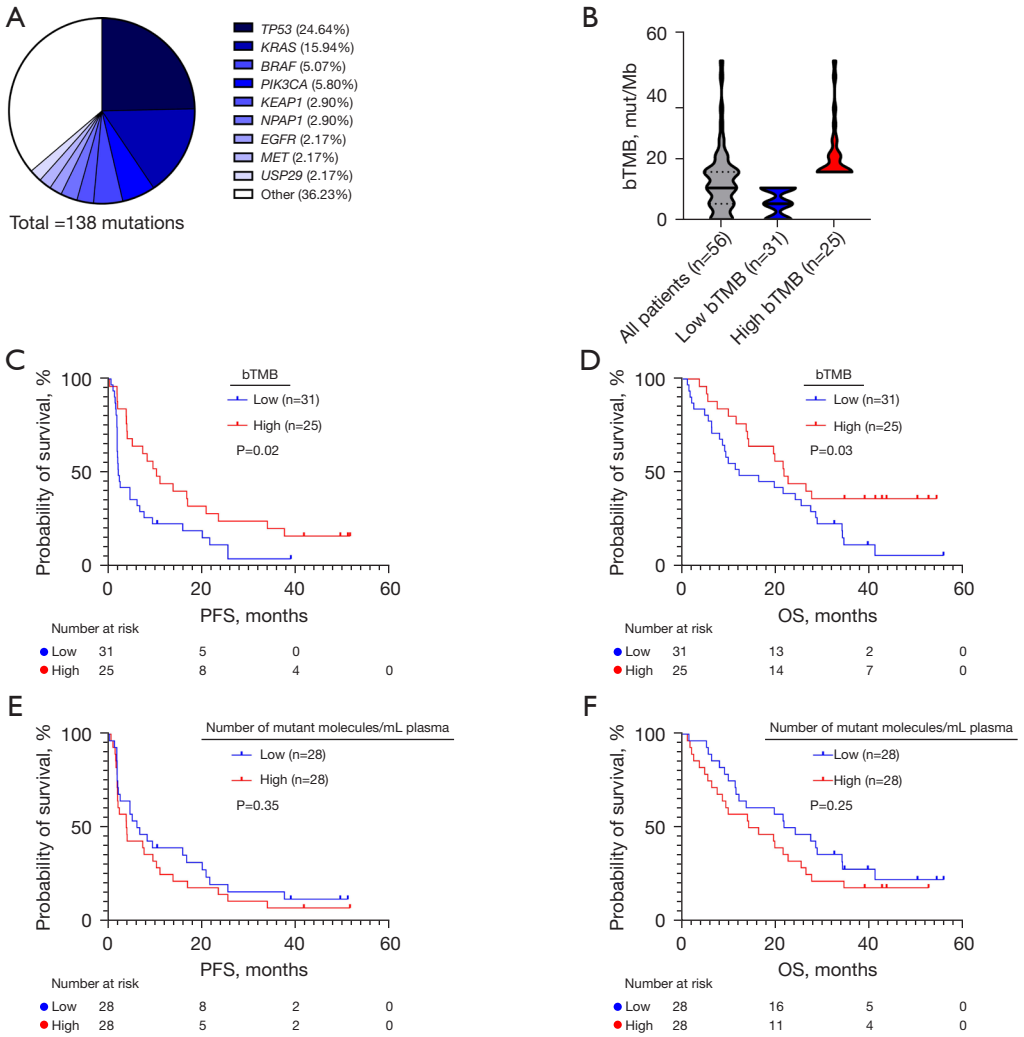

Figure 2 Overview of ctDNA sequencing in the pre-treatment sample (T_0). (A) Mutated genes identified in at least 3 patients. (B) Distribution of bTMB for all patients (grey), the low bTMB subgroup (blue) and the high bTMB subgroup (red). Patients were stratified into bTMB subgroups based on the median bTMB (10.10 mutations per Mb). (C,D) Kaplan-Meier survival analysis according to bTMB group. (E,F) Kaplan-Meier survival analysis according to the average number of mutant molecules per mL plasma. Patients were stratified based on the median (52.44 mutant molecules per mL plasma). bTMB, blood tumor mutational burden; OS, overall survival; PFS, progression-free survival; T_0, pre-treatment sample.

information about treatment response. Forty-one/56 patients (73.21%) had blood samples taken both before and after one or two cycles of treatment. Sequencing of T_x plasma samples led to the detection of mutations in 28 patients (68.29%). A total of 59 mutations in 29 different genes were detected, with the most commonly mutated genes being *TP53* (20.34%), *KRAS* (18.64%), and *BRAF* (5.08%). The patients had a median bTMB of 5.05 mutations per Mb (range, 0–40.40 mutations per Mb) (*Figure 3A*). A list of genetic alterations is provided in Table S5. Thirteen patients (31.71%) presented with undetectable ctDNA at T_x. Of these thirteen patients, seven had mutations that could be detected in the T_0 sample, and six patients had no detectable mutations before treatment initiation. The low and high bTMB groups had a similar proportion of patients without detectable ctDNA at T_x (*Figure 3B*).

We investigated if the group of patients without detectable mutations after treatment start (n=13) had a superior treatment response, compared to patients presenting with mutations (n=28). The survival analysis

Table 1 Baseline clinicopathological characteristics of the NSCLC patients based on bTMB group

Characteristics	Low bTMB (n=31)	High bTMB (n=25)	P value
Age at treatment initiation (years)			0.52
Median [range]	69 [55–88]	68 [51–78]	
Sex, n (%)			0.28
Female	13 (41.94)	15 (60.00)	
Male	18 (58.06)	10 (40.00)	
Histopathology, n (%)			>0.99
Adenocarcinoma	25 (80.65)	20 (80.00)	
Squamous cell carcinoma	6 (19.35)	4 (16.00)	
Not otherwise specified†	0 (0)	1 (4.00)	
Treatment line, n (%)			0.11
First	21 (67.74)	22 (88.00)	
Second	10 (32.26)	3 (12.00)	
Smoking status, n (%)			0.24
Active	7 (22.58)	10 (40.00)	
Former	21 (67.74)	14 (56.00)	
Never†	3 (9.68)	1 (4.00)	
Performance status, n (%)			0.54
0	7 (22.58)	8 (32.00)	
1	21 (67.74)	14 (56.00)	
2†	3 (9.68)	3 (12.00)	
Comorbidity score, n (%)			0.34
0–1	22 (70.97)	21 (84.00)	
2–5	9 (29.03)	4 (16.00)	
PD-L1, n (%)			0.74
0–49%	7 (22.58)	4 (16.00)	
50–100%	24 (77.42)	21 (84.00)	

Differences were examined using unpaired *t*-test and Fisher's exact test. †, subgroup was not included in the statistical analysis, since the number of observations did not meet the requirement for χ^2-test. bTMB, blood tumor mutational burden; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death ligand 1.

demonstrated that the patients without detectable mutations after one or two cycles of treatment had superior treatment response both in terms of PFS (20.25 *vs.* 4.18 months, HR =0.35; 95% CI: 0.16–0.72; log-rank P=0.004) and OS (28.93 *vs.* 15.33 months, HR =0.48; 95% CI: 0.22–0.98; log-rank P=0.05) (*Figure 3C,3D*). Multivariate Cox regression analysis showed an association between the absence of ctDNA and PFS (HR =0.25; 95% CI: 0.09–0.67; P=0.007) (Table S1).

The median aMM was 6.49 mutant molecules per mL of plasma (range, 0–13,400 mutant molecules per mL of plasma). Patients with a high bTMB had more significant changes in both bTMB (P<0.001, *Figure 4A*) and aMM (P=0.04, *Figure 4B*) as compared to patients with a low bTMB (P=0.24 and P=0.35). Examining the changes in aMM on an individual patient level, most patients with a

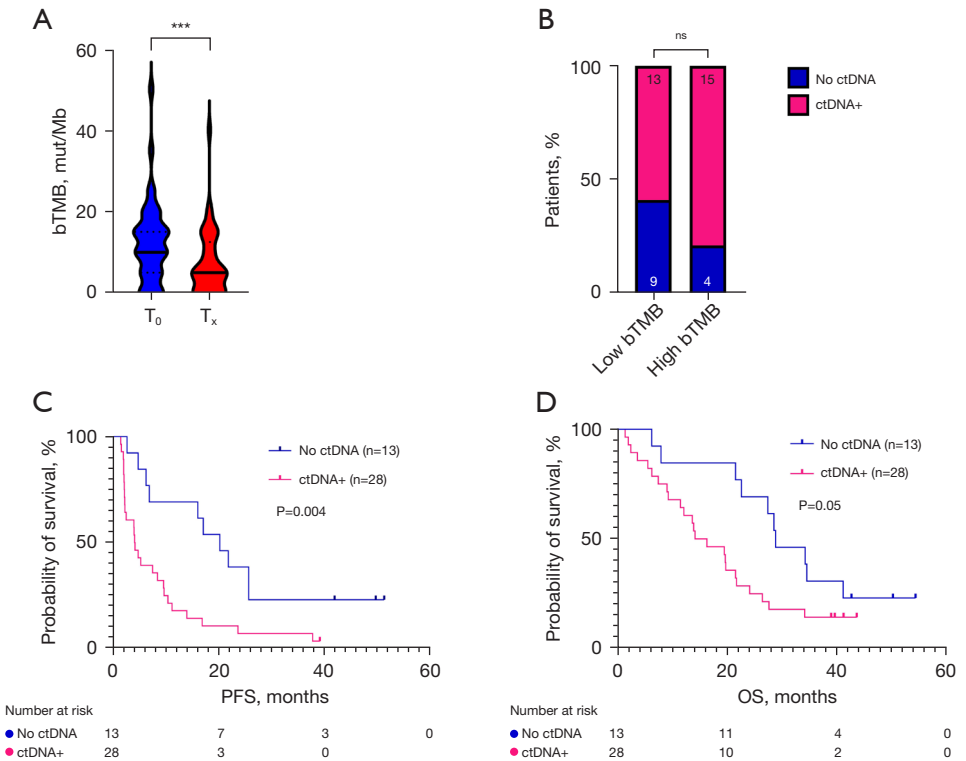

Figure 3 Overview of ctDNA sequencing after 1–2 cycles of treatment (T_x). (A) Distribution of bTMB among patients. (B) Proportion of patients in each bTMB group having an absence of ctDNA after treatment initiation. (C,D) Kaplan-Meier survival analysis according to presence or absence of ctDNA. ***, P≤0.001. bTMB, blood tumor mutational burden; ctDNA, circulating tumor DNA; ns, not significant; OS, overall survival; PFS, progression-free survival; T_0, pre-treatment sample; T_x, sample after 1–2 cycles of treatment.

high bTMB experienced a decrease in aMM in the initial weeks of treatment (*Figure 4C*). Meanwhile, patients with a low bTMB had a more varied dynamics pattern. The fraction of patients experiencing a decrease in aMM was significantly higher in the high bTMB group (P=0.03) (*Figure 4D*).

Next, we investigated if these dynamics represented response to treatment. First, we applied a conservative approach toward dynamics in aMM. We defined increase and decrease as at least 50% change in aMM from T_0 to T_x. Patients whose dynamics did not reach this threshold were included in the 'No change' group. The result of this stratification is shown in *Figure 5A,5B*. The survival analysis shows a significant overall difference in PFS and OS between the 'Decrease' (n=18), 'No change' (n=13), and 'Increase' (n=10) groups with a median PFS of 10.83 *vs.* 7.56 *vs.* 2.24 months (log-rank P<0.001) and median OS of 22.16 *vs.* 28.60 *vs.* 7.76 months (log-rank P=0.04). Interestingly, we observe little to no difference in PFS between the 'Decrease' and the 'No change' group, while the patients in the 'Increase' group experienced considerably shorter survival, both in terms of PFS and OS. Based on these results, we discarded our conservative approach for dynamics and dichotomized patients based on whether their aMM level increased (n=12) or not (n=29). Survival analysis showed that this less conservative approach still was able to identify a subgroup of patients with increasing aMM, who had very short PFS (2.19 *vs.* 11.20 months, HR =7.17; 95% CI: 3.08–16.58; log-rank P<0.001) compared to patients without any increase in aMM (*Figure 5C*). The same difference was observed in terms of OS (7.76 *vs.* 24.20 months, HR =3.05; 95% CI: 1.41–6.24; log-rank P=0.002) (*Figure 5D*). Multivariate Cox regression analysis confirmed that increasing aMM levels were associated with PFS (HR =4.60; 95% CI: 1.83–11.63; P=0.001),) and OS (HR =3.45; 95% CI: 1.55–7.40; P=0.002) (Table S1). Besides the correlation between aMM dynamics and survival, correlation between aMM dynamics and the radiological response was investigated. Using Fisher's exact test, a statistically significant correlation was

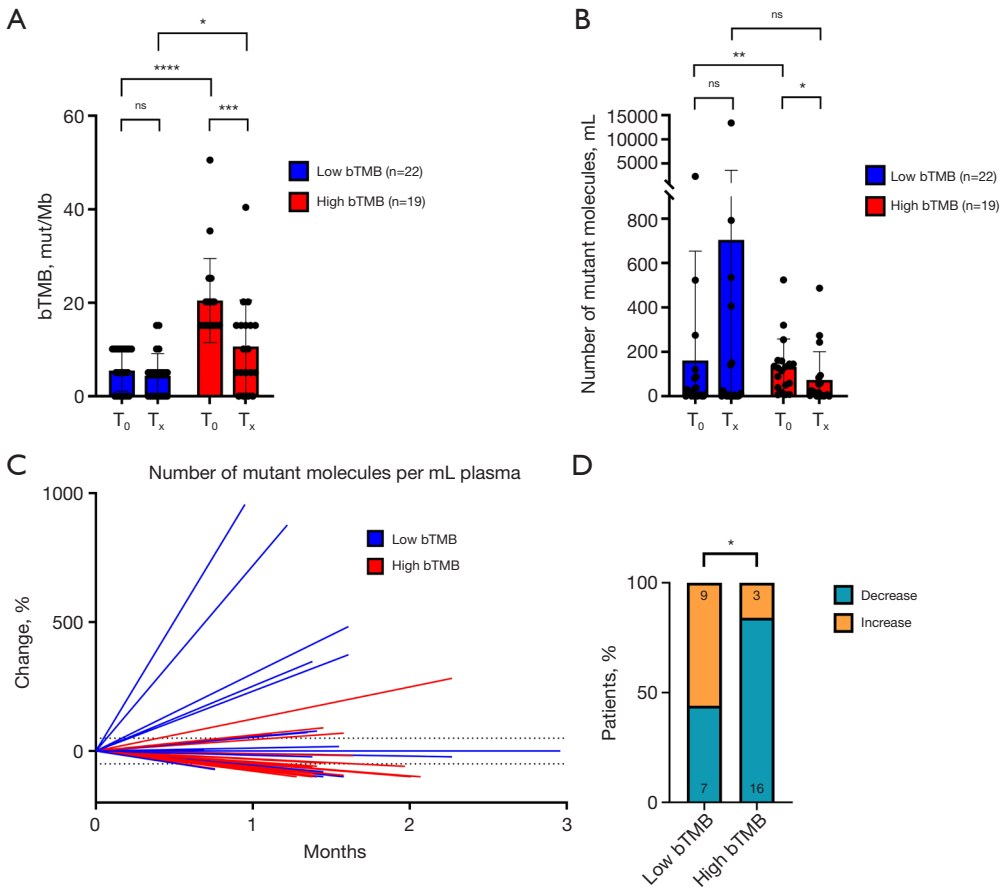

Figure 4 Dynamics in ctDNA after 1–2 cycles of treatment. (A) Changes in bTMB and (B) changes in number of mutant molecules per mL plasma according to mutational group. (C) Percent change in the number of mutant molecules per mL plasma, with blue lines representing patients with low baseline bTMB and red lines representing patients with high baseline bTMB. Dotted lines represent 50% change. (D) Proportion of patients in each bTMB group experiencing either decrease or increase in number of mutant molecules per mL. *, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001. bTMB, blood tumor mutational burden; ctDNA, circulating tumor DNA; ns, not significant; T_0, pre-treatment sample; T_x, sample after 1–2 cycles of treatment.

found between the group without increase and favorable response (complete or partial response as best overall response) (P=0.008) (Table S6).

The predictivity of the currently standard biomarker PD-L1 was evaluated in the patient cohort demonstrating a median PD-L1 TPS of 70% (range, 1–100%). We found no association between PD-L1 TPS and survival when dichotomizing based on below or above 50% TPS neither for PFS (2.56 *vs.* 5.33 months, HR =0.75; 95% CI: 0.40–1.55; log-rank P=0.51) nor OS (16.41 *vs.* 19.86 months, HR =0.66; 95% CI: 0.34–1.37; log-rank P=0.29) (Figure S2A,S2B). Next, patients were allocated into 0–49%, 50–75%, and 75–100% PD-L1 TPS groups, however, the results remained insignificant (2.56 *vs.* 4.01 *vs.* 10.42 months, log-rank P=0.32 and 16.41 *vs.* 14.24 *vs.* 23.44 months, log-rank P=0.18 for PFS and OS, respectively) (Figure S2C,S2D).

Discussion

The use of ctDNA offers a new variety of possibilities for monitoring disease and predicting response to treatment. In this study, we demonstrate that sequencing of ctDNA from plasma samples taken right before treatment start and after one or two cycles of pembrolizumab treatment contain predictive value about treatment response and survival. The presence of a high bTMB before treatment start was predictive of a longer PFS compared to patients

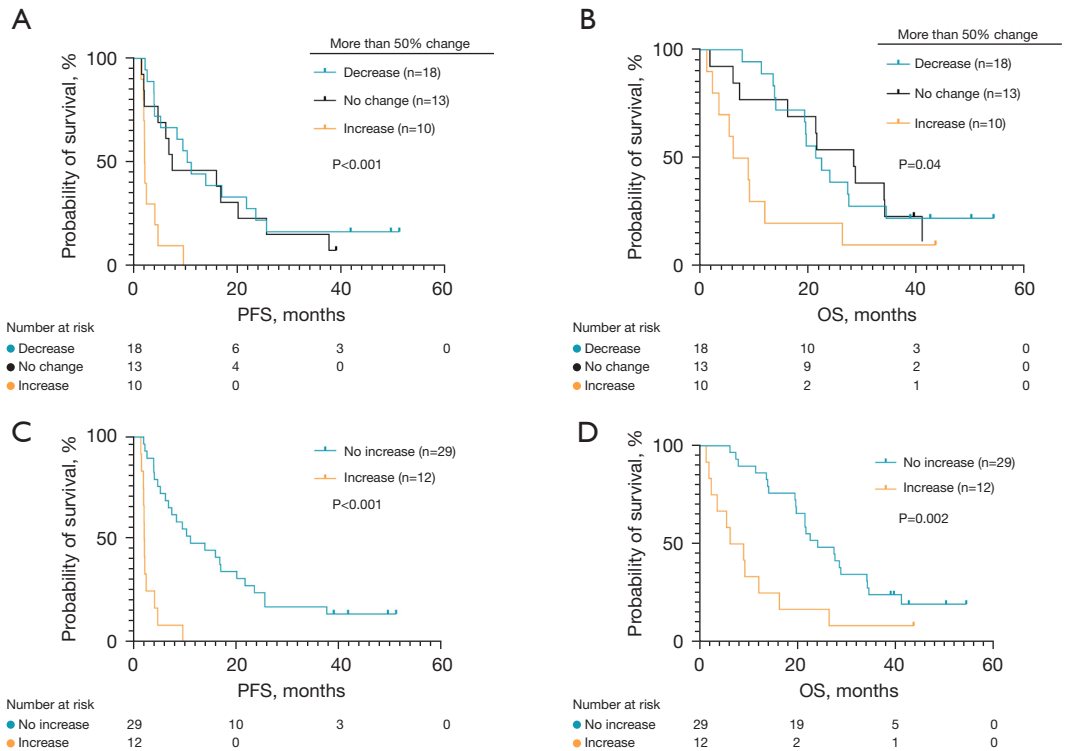

Figure 5 Dynamics in the number of mutant molecules per mL plasma. (A,B) Kaplan-Meier survival analysis according to change in ctDNA with a threshold of 50% for change. (C,D) Kaplan-Meier survival analysis for patients experiencing any increase in ctDNA. OS, overall survival; PFS, progression-free survival.

with a low bTMB. This finding is consistent with previous studies showing a high non-synonymous mutational burden is associated with response and survival in cancer patients treated with immune checkpoint inhibitors (18,19). These studies are performed on tissue biopsies, but we are able to demonstrate it in ctDNA, which is minimally invasive and circumvents the problem with tumor tissue heterogeneity.

The observed treatment effect in patients with a high mutational burden could be explained by a corresponding higher neoantigen burden. A study by Choudhury *et al.* found a linear positive correlation between the number of somatic non-synonymous mutations and the number of predicted neoantigens in bladder cancer (20). Turajlic *et al.* showed that indels in particular increased the immunogenicity of the tumor, and thereby checkpoint inhibitor response (21). A correlation between neoantigen burden and both response to immunotherapy and survival has been detected in earlier studies in lung cancers (18,22,23). The TRACERx studies have shown that NSCLC patients have a high degree of intratumoral heterogeneity, which affects the response to treatment (22,24-27). A study by McGranahan *et al.* found a positive correlation between high neoantigen burden and overall survival in lung adenocarcinoma patients, where this effect was most pronounced in tumors with a homogenous neoantigen load (22), similarly Zhou *et al.* demonstrated that high heterogeneity in ctDNA was associated with poor response to immune checkpoint inhibitors (28). Others found no correlation between mutational and neoepitope load and longer survival (29,30). However, these studies were performed on tissue biopsies and peripheral blood mononuclear cells, and our study was performed on ctDNA, which may explain the discrepancy.

Our study shows that the analysis of the T_x samples collected after one or two cycles of treatment detected two important factors, when identifying which patients will benefit from treatment: the absence of ctDNA and the dynamics in ctDNA level during the initial weeks of treatment. Patients with undetectable ctDNA after treatment initiation had significantly longer PFS and OS. The absence of ctDNA can be explained by clearing due to effective treatment, or due to the concept of 'non-shedding',

which describes patients without detectable mutations in neither the pre- nor the post-treatment initiation sample. This may happen due to low tumor burden, less mitotic activity, or limited vascularization, among others (31,32). Previous studies have found both clearing and non-shedding to be correlated with survival (33-36). Our group has earlier shown that some patients clear their ctDNA at a slower pace than others and that survival is not dependent on fast clearing (36). Therefore, we turned our focus to the dynamics in ctDNA during the initial weeks of treatment. We discovered a patient subgroup with an increase in aMM after one or two cycles of treatment who had significantly inferior survival both in terms of PFS and OS. All patients experiencing any increase in aMM after treatment progressed within 10 months after treatment initiation. The increase in aMM seems to be an important biomarker for identifying patients with no benefit from treatment with pembrolizumab. This information could be vital for clinicians, so patients can change treatment or be subjected to combination therapy, instead of continuing treatment without effect.

Recent studies by Weber *et al.* and Vega *et al.* investigated the relationship between ctDNA dynamics and response to immunotherapy (33,37). The study by Weber *et al.* also used the average number of mutant molecules per mL of plasma as a measure of tumor burden while Vega *et al.* quantified the ctDNA level using the maximum variant AF. Weber *et al.* stratified patients in a two-level model based on a 50% decrease in ctDNA levels and showed inferior survival for patients with less than 50% decrease (33). The study by Vega *et al.* employed a three-level model for stratifying patients into three groups; based on 50% increase, 50% decrease or less than 50% change in ctDNA level and observed an association between the subgroups and survival (37). In our study we first stratified patients based on a three-level approach similar to the study by Vega *et al.*: ≥50% increase, ≥50% decrease, or no change in ctDNA (<50% change). An interesting similarity was observed between patients with a high level of decrease and those with less or no change at all. Furthermore, we found that a threshold of 50% change did not add value to the stratification of patients.

Other studies have similarly shown an association between decreasing ctDNA levels and response, with the variant AF or the number of non-synonymous mutations per Mb being utilized as a measure for ctDNA levels (38-41). In contrast, our study uses the average number of mutant molecules per mL of plasma, which takes the isolated DNA mass, the volume of plasma, and the AF into account, using a commercially available targeted NGS panel. Besides an association between decreasing ctDNA levels and PFS and OS, this approach was able to identify a subgroup of patients with early increasing ctDNA levels who all progressed within 10 months after treatment initiation.

Despite the significant results presented, the study still faced limitations. A limitation is the number of patients (n=56), which restricted the stratification of patients into subgroups in our statistical analyses. Furthermore, there was variability in the duration of treatment at the time of T_x sampling (range, 0.69–2.96 months). Thus, the presented results should be interpreted with caution and should be repeated and validated in a larger more uniform cohort. However, this study represents a real-world cohort, which enhances the external validity of our findings. Additionally, all patients were treated with the same checkpoint inhibitor, pembrolizumab, with the majority receiving pembrolizumab in a first-line setting. We used a commercially available panel for targeted NGS, which contains 197 genes chosen based on their relevance in lung and colorectal cancer (42). We detected mutations in 47 out of 56 patients (83.93%) in pre-treatment ctDNA using this panel, thus the targeted NGS approach is suitable for monitoring NSCLC patients. Additionally, the usage of a commercial panel allows for easy implementation and enables comparison between studies.

Conclusions

This study shows that the presence of a high bTMB before treatment initiation is predictive of superior survival, while the pre-treatment number of mutant molecules per mL of plasma contains no information regarding response and survival. The absence of ctDNA after treatment initiation correlates with longer survival, while an increase in the ctDNA level is correlated with inferior survival. Our results show that ctDNA monitoring identifies early predictive factors for response and survival, which is vital for identifying patients without response to treatment.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-818/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and all patients provided written informed consent. The study was approved by the regional ethics committee of Region Midt (Approval No. 1-16-02-211-16).

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