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Phase II study of nivolumab in patients with genetic alterations in DNA damage repair and response who progressed after standard treatment for metastatic solid cancers (KM-06)

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ABSTRACT

Background Immune-modulating antibodies targeting programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1) have demonstrated promising antitumor efficacy in various types of cancers, especially highly mutated ones. Genetic alterations in DNA damage response and repair (DDR) genes can lead to genetic instability, often accompanied by a high tumor mutation burden (TMB). However, few studies have validated the aberration of DDR genes as a predictive biomarker for response to immune-modulating antibodies.

Methods The KM-06 open-label, multicenter, single-arm, phase II trial evaluated the safety and efficacy of nivolumab in refractory solid cancers with DDR gene mutations assessed by clinically targeted sequencing. Nivolumab (3 mg/kg) was administered every 2 weeks until disease progression, unacceptable toxicity, or for 24 months. The primary endpoint was the objective response rate (ORR) as per RECIST V.1.1 criteria.

Results A total of 48 patients were enrolled in the study (median age 61, 58.3% male). The most common cancer type was colorectal cancer (41.7%), followed by prostate and biliary tract cancer (8.3% each). Eight patients achieved a partial response as their best overall response, resulting in an ORR of 17.8%. The disease control rate was 60.0%. The median progression-free survival was 2.9 months. Treatment-related adverse events of any grade and grade ≥3 occurred in 44 (91.7%) and 4 (8.3%) patients, respectively. Clinically targeted sequencing data inferred both TMB and microsatellite instability (MSI). Using a TMB cut-off of 12 mut/Mb, there were significant differences in overall survival (p=0.00035), progression-free survival (p=0.0061), and the best overall response (p=0.05). In the RNA sequencing analysis, nivolumab responders showed activation of the interleukin signaling pathway. Patients who experienced early progression presented high epithelial-mesenchymal transition signaling pathway activation. The responders exhibited a marked increase in PD-1−/Ki67+CD8T cells at the early stage of treatment (C3D1) compared with non-responders (p=0.03).

Conclusions In this phase II trial, nivolumab demonstrated moderate efficacy and manageable toxicity in patients with solid cancer harboring DDR gene mutations. A high TMB (>12 mut/Mb) and MSI score (>2.5) determined through clinically target sequencing presented significant discriminatory power for the nivolumab response.

Trial registration number NCT04761744.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Prior research indicated that immune checkpoint inhibitors (ICIs) are effective against tumor mutation burden (TMB)-high cancers. It was known that mutations in DNA damage response and repair (DDR) genes could lead to a high TMB, potentially affecting cancer stability. Yet, the role of DDR gene mutations as reliable indicators for predicting the response to ICI was not well established.

WHAT THIS STUDY ADDS

⇒ Nivolumab, a PD-1 targeting antibody, has moderate efficacy and manageable toxicity in treating solid cancers with DDR gene mutations. A high TMB>12 mut/Mb determined through clinically target sequencing is a significant indicator of a positive response to nivolumab.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The findings encourage further research into the role of TMB as predictive biomarkers for immunotherapy response, in solid cancers with DDR gene mutations. The results may guide oncologists in selecting patients who are likely to benefit from nivolumab treatment by considering TMB assessed by clinically targeted sequencing.

INTRODUCTION

The efficacy of immune checkpoint inhibitors (ICIs), including antibodies that

block programmed cell death protein-1 (PD-1) and programmed cell death protein ligand-1 (PD-L1), has been demonstrated in various solid tumors. Nivolumab, the first-in-human IgG4 PD-1 ICI antibody, has been approved by the Food and Drug Administration for many cancers. This includes lung cancer, melanoma, urothelial carcinoma, and esophageal cancer. The latter two cancers were previously categorized as refractory to cytotoxic chemotherapy.[1–3]

Despite the prolonged response and acceptable toxicities of ICIs, most patients fail to experience these advantages, leading to increased focus on discovering predictive biomarkers.[4 5] The investigation into predictive biomarkers for ICI efficacy has expanded from studying cell surface markers[6] and the tumor microenvironment (TME)[7] to exploring tumor DNA[8] and systemic factors within the host.[9]

Tumor mutational burden (TMB) is a measurement that quantifies the number of mutations/Mb harbored by tumor cells. The TMB has been extensively studied as a predictive biomarker of ICI response.[8] A pooled analysis of 24 tumor types has demonstrated that tumors with high TMB tend to present a more effective clinical response to ICI than tumors with low TMB.[10] This phenomenon is theoretically based on a high burden of immunogenic neoantigens and host T cell recognition, which can effectively activate ICI treatment.[11]

Genetic alterations in DNA damage response and repair (DDR) genes can lead to genetic instability and frequent pathologic mutations, often accompanied by a high TMB.[12] In contrast to TMB, which requires exome-wide sequencing for accurate determination, DDR gene mutations can be easily confirmed through targeted panel sequencing. Despite the potential of such biomarkers, a limited number of studies have validated the aberration of DDR genes as a predictive biomarker for response to ICIs.

Here, we discuss the results of the KM-06 clinical trial, which investigated the efficacy of nivolumab treatment in patients with solid tumors containing DDR gene mutations detected through targeted sequencing.

METHODS

Study design and patients

This is a multicenter, single-arm, phase 2 study conducted as a clinical substudy of the K-MASTER project, a nationwide, government-funded precision medicine initiative.[13] Eligible patients were ≥20 years of age with histologically or cytologically confirmed solid cancer refractory to one or more chemotherapies. The cancer should have been confirmed to have a DNA damage repair pathway aberration by next-generation sequencing (NGS). Eligible patients had at least one measurable disease, an Eastern Cooperative Oncology Group performance status of 0 or 1, and adequate organ function. Any prior treatment with anti-PD1 or PD-L1 inhibitors was not permitted, and prior immunosuppressive treatment or the last dose of chemotherapy should not have been administered within 28 days before the first dose of the study drug.

Treatment and evaluation

Nivolumab (3 mg/kg) was administered intravenously every 2 weeks until disease progression, unacceptable toxicity, patient refusal, or for 2 years of treatment. Dose modification was not allowed; however, a dose delay of up to 4 weeks at the investigators' discretion was permitted for clinically significant events. Response assessment involved CT or other imaging modalities according to Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1. every 6 weeks. When treatment ended, the patients were followed up every 3 months concerning disease status, treatment, and survival. The medical histories of all patients were obtained before treatment, including physical examination, complete blood count with differential count, serum chemistry, electrolytes, coagulation, carcinoembryonic antigen, thyroid function test (thyroid-stimulating hormone and free thyroxine), urinalysis, ECG, chest X-ray, CT scan, and other scans if clinically indicated. Adverse events were assessed every cycle according to the National Cancer Institute Common Terminology Criteria for Adverse Events, V.4.03.

Biomarker analysis

During the screening period, tumor tissues or blood samples were obtained for exploratory biomarker analysis. Targeted sequencing based on NGS was performed with the K-MASTER Korean solid cancer genome analysis research project.[13]

Next-generation sequencing

Targeted sequencing using tumor tissues was performed using CancerSCAN (Samsung Genome Institute, Seoul, Korea). In patients whose tumor tissues were not available, 10 mL of whole blood was collected with Cell-Free DNA BCT for circulating tumor DNA preparation and analysis by the AXEN Cancer Panel (Macrogen, Seoul, South Korea). The sequential blood sample was collected within 24 hours before treatment, before the start of the second cycle, at the time of response evaluation, and at the end of treatment.

Genomic DNA from formalin-fixed paraffin-embedded (FFPE) samples or plasma was extracted using the QIAamp FFPE tissue kit (Qiagen, Hilden, Germany) or the QIAamp circulating nucleic acid kit (Qiagen), respectively. Cell-free DNA purity was measured using a High Sensitivity DNA Kit and a 2100 Bioanalyzer instrument (Agilent Technologies). When required, additional purification was performed using an Agencourt AMPure XP device (Beckman Coulter, Brea, California, USA) to remove the contaminating nucleic acid further. Centrally isolated genomic DNA samples that underwent quality control were sent to the K-MASTER genomic analysis laboratories. K-MASTER used two previously established tissue-based NGS panels (FIRST and CancerSCAN) to detect major genomic aberrations, including mutations,

circulating nucleic acids, and small insertions and deletions in cancer-related genes. CancerSCAN has been further upgraded to K-MASTER V.1.0 and V.1.1

Mutational calls

Exome sequencing reads were aligned to the human reference genome (hg3) using the Burrow-Wheeler Aligner. The initial aligned BAM files were subjected to preprocessing steps, including sorting, removal of duplicated reads, local realignment around small indels, and recalibration of base quality scores using SAMtools, Picard, and the Genome Analysis ToolKit. Somatic variant callings were performed using MuTect2. We used the 1000 Genomes, gnomAD, and dbSNP datasets to provide a reference for known polymorphic sites. Each mutation was further annotated using vcf2maf. Mutations with the following criteria were used for downstream analysis: "PASS" in the "FILTER" column, minimum coverage depth of 50, and tumor alteration read count >5. To distinguish somatic mutations from germline mutations, we used the 1000 Genomes, gnomAD, and dbSNP datasets as the reference databases for recognized polymorphic sites. The mutations that had not been reported before were exclusively incorporated.

Whole-transcriptome sequencing

RNA sequencing reads were mapped to the human genome reference (hg38) using STAR aligners. Each aligned read was analyzed using featureCounts from Subread. The gene expression levels were quantified as fragments per kilobase of transcript per million by bioinfokit.

Microsatellite instability status

We investigated the stability of microsatellite regions using microsatellite instability (MSIsensor2), and the somatic status of MSI events in each tumor was applied to represent the MSI scores.

Identification of molecular and clinical features using multivariable model analysis

We applied a gradient-boosting machine algorithm to predict the clinical response to nivolumab treatment based on multiple layers of molecular and clinical variables. The input variables of GBM consist of somatic mutations in cancer-driver genes, mutational signature activities, MSIseq status, MSIsensor scores, TMB (nonsynonymous and synonymous), and histopathological characteristics. We used the xgboost package to build a gradient-boosted tree algorithm, and all training samples were leveraged to build a predictive model with 4-fold cross-validation and performed with a maximum tree depth of 3, eta of 0.3, nround of 200, and a subsample of 0.8. All other parameters were kept as default values.

Flow cytometry (fluorescence-activated cell sorting) analysis

From the blood collected during the study period, peripheral blood mononuclear cells (PBMCs) were isolated. Fluorescence-activated cell sorting (FACS) analysis was conducted based on a previously used and validated protocol in the Department of Biochemistry and Molecular Biology, Korea University College of Medicine.[14] FACS data obtained using a BD FACSCanto device were analyzed using FlowJo software V.10.

Statistical analyses

The sample size of this study was calculated using the Simon two-stage optimal design. The target response rate was 25%. A rate ≤10% was considered a failure, allowing early termination of any ineffective treatment early in the study. With a two-sided type I error of 5% and a power of 0.8, the planned study proceeded in two steps. If a tumor response occurred in at least two patients after the first 18 patients were listed, the study proceeded to the second stage with 25 additional patients. A total of 43 patients were required, and enrolment of 48 patients was planned, considering a drop-out rate of 10%.

This study's primary endpoint is to evaluate the object response rate (ORR) of disease assessed by RECIST V.1.1. ORR was defined as the proportion of patients with a complete response (CR) and a partial response (PR). The secondary endpoints included ORR assessed by immune-related RECIST (irRECIST), disease control rate, progression-free survival (PFS), overall survival (OS), toxicity profile, and exploratory biomarker analysis. DCR was defined as the proportion of patients with CR, PR, or stable disease (SD). PFS was calculated from the first date of nivolumab administration to the date

Table 1 Baseline clinical characteristics of the 48 patients

Clinical characteristics	N (%)
Age (years), median (range)	61.0 (27–84)
Sex, n (%)	
Female	20 (41.7)
Male	28 (58.3)
Cancer diagnosis	
Colorectal cancer	20 (41.7)
Prostate cancer	4 (8.3)
Biliary tract cancer	4 (8.3)
Ovarian cancer	3 (6.3)
Stomach cancer	3 (6.3)
Breast cancer	2 (4.2)
Sarcoma	2 (4.2)
Others	10 (20.8)
Previous lines of therapy	
1	10 (20.8)
2	16 (33.3)
3	12 (25.0)
≥4	4 (8.3)
NE	5 (10.4)

NE, not evaluable.

of disease progression or death from any cause. OS was defined as the duration from the first date of nivolumab administration to the date of death from any cause. A two-sided p<0.05 was considered to indicate statistical significance. The statistical analyses were performed using PASS V.12.0.2 (2013, NSCC).

RESULTS

Patient characteristics

From June 2019 to January 2021, 48 patients were enrolled at 11 hospitals in South Korea. The baseline clinical characteristics of the 48 patients are summarized in table 1. The median age was 61 years (range 27–84), and 58.3% were male. The most common cancer diagnosis was colorectal cancer (n=20, 41.7%), followed by prostate and biliary tract cancer (each n=4, 8.3%). Thirty-two patients (66.6%) were treated with more than two lines of chemotherapy for palliative purposes before study enrolment. *BRCA2* was the most commonly mutated gene associated with the DDR pathway (n=18, 37.6%), followed by *ATM* (n=10, 20.8%), and *BRCA1* (n=8, 16.7%). Online supplemental table 1 contains the list of genetic mutations used as criteria for study participation, and the allele frequencies (AF) corresponding to different mutation types are presented in online supplemental figure 1.

Treatment response to nivolumab

Among the 48 patients, 45 completed the study treatment with at least one or more response evaluations. The treatment response and duration are provided in figure 1A. Eight patients achieved a PR as their best treatment response, resulting in an ORR of 17.8% for nivolumab treatment. No patient achieved a CR. The DCR was 60.0%, with 19 (42.2%) patients with SD as their best response. The ORR and DCR assessed by irRECIST were 13.3% and 42.2%, respectively. The median follow-up duration was 8.3 months (0.3–26.6), and the median PFS (mPFS) was 2.9 months (0.3–23.1). Patients who achieved PR or SD for their best response (responders) had a longer PFS than those who did not (non-responders). The difference between these patients was statistically significant (mPFS 5.7 vs 2.3 months, p<0.0001) (figure 1B). During the follow-up period, 16 deaths occurred, with a median OS of 7.8 months (1.9–16.8). Nivolumab was discontinued mainly due to disease progression (n=35, 72.9%). Four (8.9%) patients continued treatment for more than 2 years.

Safety analysis

Treatment-related adverse events (TRAEs) with nivolumab are provided in table 2. TRAEs of any grade were observed in 44 patients (91.7%). Common TRAEs of any grade included pruritus (n=7, 14.6%), increased aspartate transaminase (AST) or alanine transaminase (ALT) (n=4, 8.3%), skin rash (n=4, 8.3%), hypothyroidism (n=4, 8.3%), and fatigue (n=4, 8.3%). Grade 3 or 4 TRAEs, classified as severe adverse events, were

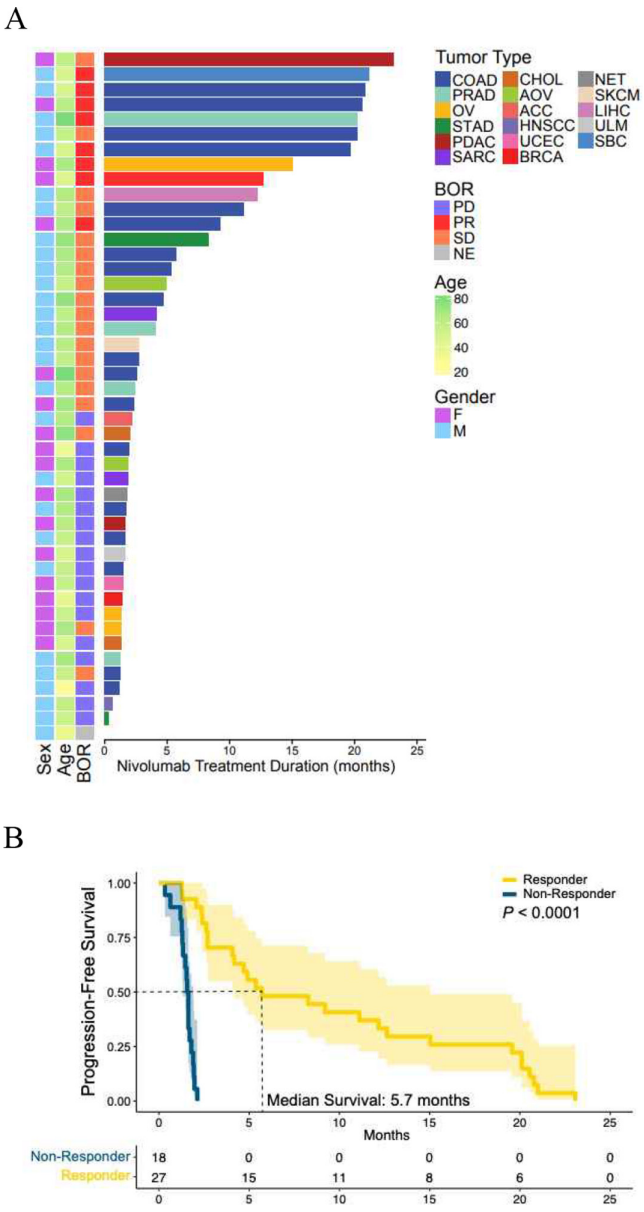

Figure 1 (A) Swimmer plot of patients who received nivolumab. Each lane represents an individual patient's tumor type, best objective response, sex, age, and treatment duration. (B) Progression-free survival of patients based on the RECIST criteria response. PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease.

reported in four patients (8.3%), involving increased AST or ALT (n=2, 4.2%) and increased alkaline phosphatase gamma-glutamyl transferase (n=1, 2.1%), and acute kidney injury (n=1, 2.1%). One patient who experienced a severe increase in AST and ALT discontinued treatment due to toxicity. No deaths were related to TRAEs.

Biomarker analysis

Targeted gene sequencing

We previously categorized the patients who participated in the trial into non-responders and responder groups based on their best response, with responders achieving PR or SD. The genomic landscape analysis, including

Table 2 Treatment-related adverse events

Event	All patients (N=48, 100%)	
	Any grade	Grade≥3
Any TRAE	44 (91.7%)	4 (8.3%)
Pruritus	7 (14.6%)	0
Increased AST or ALT	4 (8.3%)	2 (4.2%)
Skin rash	4 (8.3%)	0
Hypothyroidism	4 (8.3%)	0
Fatigue	4 (8.3%)	0
Increased ALP or GGT	3 (6.3%)	1 (2.1%)
Hyperbilirubinemia	2 (4.2%)	0
Nausea	2 (4.2%)	0
Dizziness	2 (4.2%)	0
Adrenal insufficiency	2 (4.2%)	0
Myalgia	2 (4.2%)	0
Itching sense	2 (4.2%)	0
Sore throat	1 (2.1%)	0
Hyperglycemia	1 (2.1%)	0
Pneumonitis	1 (2.1%)	0
Acute kidney injury	1 (2.1%)	1 (2.1%)
Arthralgia	1 (2.1%)	0
Chills	1 (2.1%)	0
Conjunctivitis	1 (2.1%)	0
Abdominal pain	1 (2.1%)	0
Dry mouth	1 (2.1%)	0
Eosinophilia	1 (2.1%)	0
Gait disturbance	1 (2.1%)	0
Peripheral neuropathy	1 (2.1%)	0
Thrombocytopenia	1 (2.1%)	0
Hypoalbuminemia	1 (2.1%)	0
Hypocalcemia	1 (2.1%)	0
Hypokalemia	1 (2.1%)	0
Abdominal pain	1 (2.1%)	0
Palmar-plantar erythrodysesthesia syndrome	1 (2.1%)	0
Shingles	1 (2.1%)	0
Upper respiratory infection	1 (2.1%)	0
Vomiting	1 (2.1%)	0
Wound infection	1 (2.1%)	0

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transferase; TRAE, treatment-related adverse event.

the assessment of responder status, is provided in figure 2. Responders showing significant tumor volume reduction (on the right side of figure 2 exhibited a higher frequency of mutations in mismatch repair, homologous recombination, and cancer gene census). To detect potential predictive biomarkers for the nivolumab response, we calculated TMB using data from targeted sequencing (figure 3). We first compared TMB between responders and non-responders (figure 3A). No statistical difference exists in TMB between the two groups (p=0.104). Participants were reclassified based on a threshold of TMB 12, which had the lowest p value among the predictive criteria (figure 3B). TMB exceeded 12 for 26 patients (high TMB), while 17 patients had TMB≤12 (low TMB). The median OS of patients with high TMB and low TMB was 44.03 and 7.93 months, respectively (figure 3C). The HR for OS was 0.251 (p=0.00035). The mPFS of 3.43 months for patients with high TMB was significantly longer than that of patients with low TMB (1.8 months, p=0.0061) (figure 3C). Patients with high-TMB displayed a greater decrease in tumor volume (figure 3E). A similar pattern was evident in the MSK-IMPACT dataset (mPFS for ICI, $p=8.22\times10^{-13}$) (figure 3F). When analyzing the genomic and clinical data, the TMB threshold 12 exhibited the lowest p value in discrimination for both PFS and BOR (figure 3G). Signature HR deficiency in non-responders showed the highest feature importance score, followed by the TP53 mutation in responders (figure 3H).

RNA analysis

The tumor samples from 33 participants (13 non-responders and 20 responders) were subjected to RNA analysis (figure 4). Among them, the responder group exhibited increased activation of the pathways regarding interleukin signaling (figure 4A) and ERBB2/3 (figure 4B) with marginal significance (p=0.05 and p=0.493, respectively). The non-responder group displayed statistically significant activation of the epithelial-mesenchymal transition (figure 4C) and enhancer of zeste homolog 2 (EZH2) target (figure 4D) pathways (p=0.0432 and p=0.014, respectively). Gene set enrichment analysis results for each pathway are provided in online supplemental figure 2.

FACS analysis

Flow cytometry of PBMCs was performed on samples collected from 32 participants (10 non-responders and 22 responders) at baseline and in the early phase of treatment (after two cycles). Figure 5 denotes the differences between non-responders and responders. No significant difference exists in baseline PD-1$^+$/Ki-67$^+$ CD8 T cell levels between the two groups (0.08±0.04 vs 0.38±0.34, p=0.5478; figure 5A). The change from baseline to the early phase of nivolumab treatment showed minimal differences (0.06±0.032 vs 0.37±0.34, p=0.5423; figure 5B). However, among responders, a smaller proportion of PD-1$^-$/Ki-67$^+$ CD8 T cells was observed at baseline (0.34±0.1 vs 0.16±0.08, p=0.0447; figure 5C). A significant increase in PD-1$^-$/Ki-67$^+$ CD8 T cell levels was observed after two cycles of nivolumab treatment (0.25±0.06 vs 0.28±0.05, p=0.03; figure 5D). Detailed statistical values are presented in online supplemental table 2.

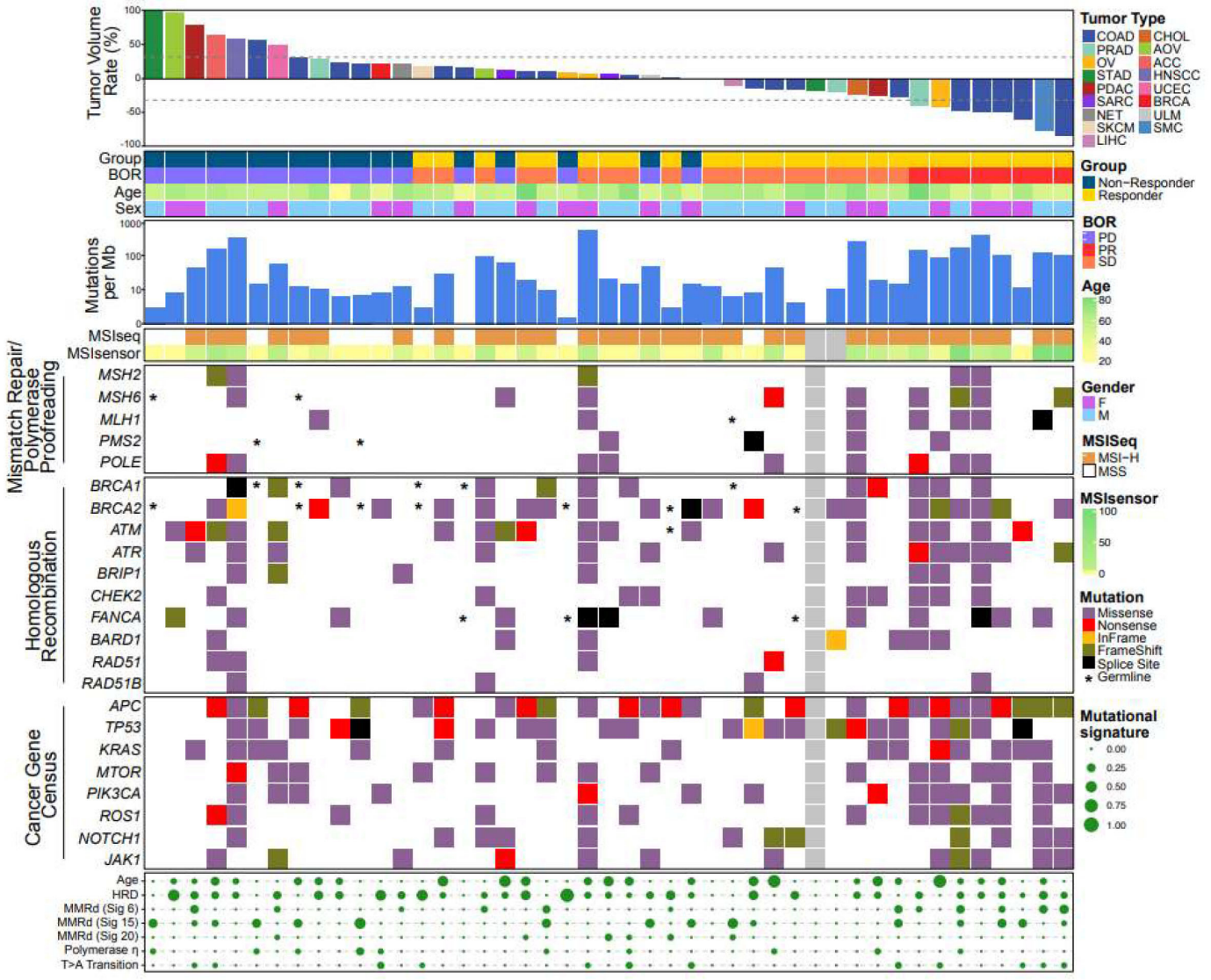

Figure 2 Molecular landscape of solid tumor patients who received nivolumab. The top panel represents the maximum tumor volume changes during nivolumab treatment based on RECIST criteria. The upper middle panel depicts the treatment response group, best objective response, age, and sex. The second middle panel demonstrates tumor mutational burden (TMB) per megabase, followed by MSIseq status and MSIsensor score. The fourth middle panel represents major somatic mutations in mismatch repair, homologous recombination-associated pathways, and cancer census genes. The bottom panel exhibits mutational signature activities. MSI, microsatellite instability; PR, microsatellite instability; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease

DISCUSSION

In this multicenter, single-arm, phase 2 study, nivolumab showed a promising clinical response with manageable toxicity in patients with solid cancer harboring DDR pathway aberrations. The ORR and mPFS were 17.8% and 2.9 months, respectively. Four patients continued nivolumab treatment with a durable response at the end of the study. Patients with TMB>12 displayed greater decreases in tumor volume than those with TMB<12.

Efforts to discover predictive biomarkers for predicting the response of ICI have been ongoing for over a decade. These efforts have expanded the scope of simple blood tests, such as lymphocyte ratio,[15] to include PD-L1 expression scores[16] and TMB or MSI from gene sequencing.[8] However, these biomarkers have failed to precisely predict ICI treatment responses and have proven challenging in clinical practice due to their cost and complexity. PD-L1 IHC is the most logical and relatively straightforward measure. Accordingly, it has been widely used. Several clinical trials have grouped patients based on PD-L1 expression and proceeded with treatment.[17 18] Nevertheless, the predictive power has not been perfect, as a few PD-L1-negative patients still showed efficacy with ICIs.[19] The differences in testing methods and the low reproducibility of results make PD-L1 IHC unsuitable as a reliable predictive biomarker.[20]

The other most commonly used biomarker is TMB. Whole-exome sequencing is essential to precisely calculate TMB.[21] Due to the high cost and turnaround time constraints, TMB information from targeted panel

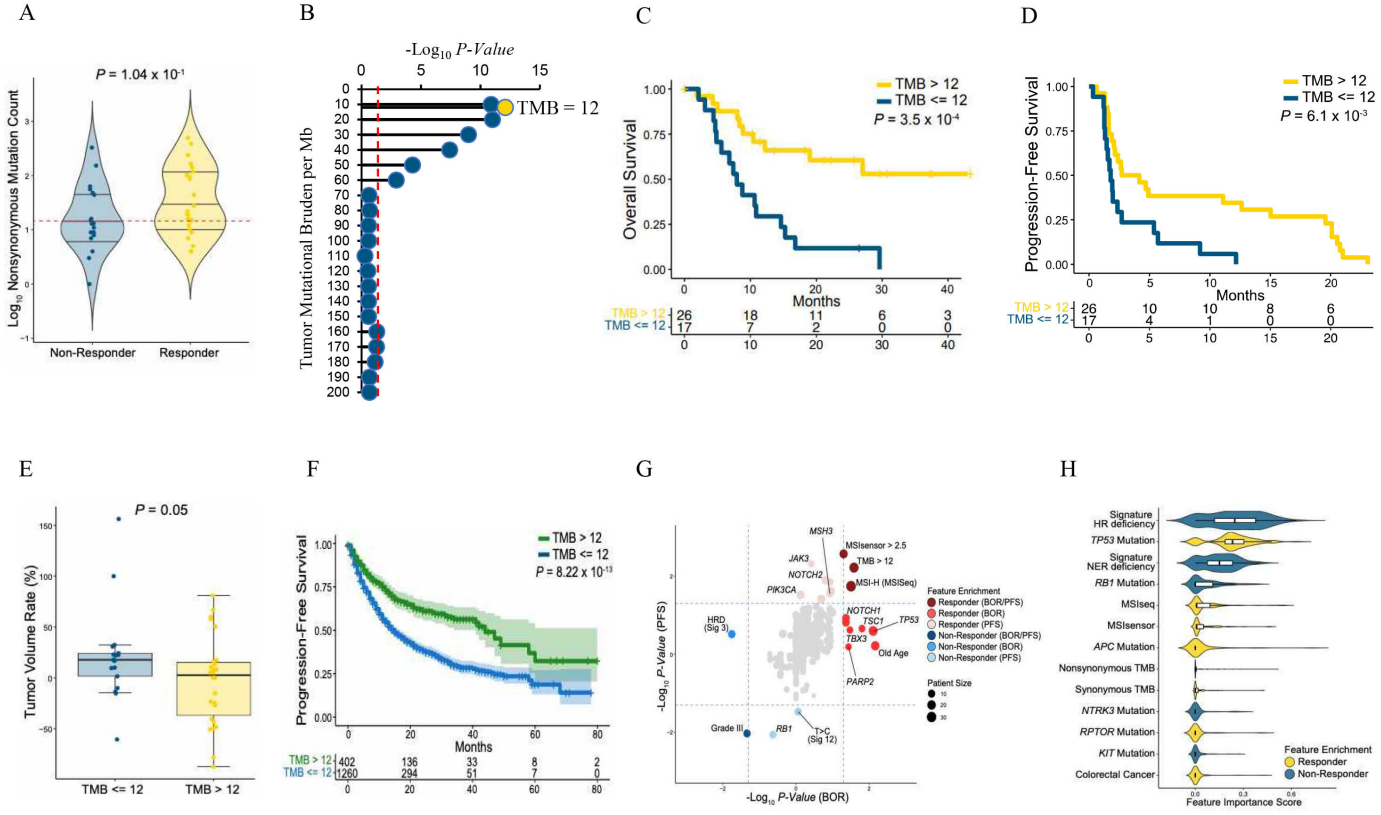

Figure 3 (A) Comparison of tumor mutational burden (TMB; non-synonymous mutations) between responder and non-responder groups. (B) Identification of an optimal TMB cut-off for distinguishing responders from non-responders to nivolumab treatment. (C) Overall survival of patients based on the optimal TMB cut-off. (D) Progression-free survival of patients based on the optimal TMB cut-off. (E) Comparison of tumor volume changes based on the optimal TMB cut-off. (F) Validation of the optimal TMB cut-off using the MSK-IMPACT cohort who received immune checkpoint blockades. (G) Identification of molecular and clinical correlates that are significantly associated with progression-free survival (y-axis) and the best objective response (x-axis) in response to nivolumab treatment. (H) Feature importance scores of each molecular and clinical feature against nivolumab treatment using a gradient-boosting machine algorithm. The model adopted a bootstrapping strategy 100 times to obtain a robust evaluation of the predictive features.

sequencing has gained a significant role in clinical settings over whole-exome sequencing. Since Chalmers *et al* reported a high correlation between TMB measured by whole exome sequencing and comprehensive genomic profiling (R^2=0.74),[21] the majority of targeted panels offer TMB information, and a few (FoundationOne CDx) have been approved as a companion diagnostic for ICIs.[22 23] Nevertheless, concerns regarding their credibility persist. A study reported a strong correlation in inferred TMB values from targeted sequencing at high TMB (eg, ≥20 mut/Mb); however, there is a weak correlation at intermediate levels. TMB was overestimated in tumors with low-frequency mutation.[24] Various factors, including tumor cell content, sample preparation, sequencing coverage, panel size, and bioinformatics pipeline, influence the determination of TMB through targeted sequencing.[25] Varying testing platforms are one of the key factors that could potentially limit the standardization of TMB measurement.

Contrary to the initial intent of the clinical study, the DDR gene mutation itself did not guarantee a favorable response to nivolumab. Since all patients possessed mutations in both the mismatch repair and HRD pathways, it was challenging to determine which factor had a more significant impact on the ORR. Instead, we propose a new criterion for TMB 12 through computational and statistical postanalyses. When patients were stratified based on TMB>12, the predictive power for PFS and BOR was superior to other single gene mutations and even to the well-known marker, MSI-H. Our results, with a significant colorectal cancer cohort (41.7%), resonate with prior studies on ICIs in TMB-high solid tumors. The Targeted Agent and Profiling Utilization Registry (TAPUR) study noted a 31% DCR (p=0.04) for pembrolizumab in metastatic colorectal cancer with high TMB,[26] reflecting moderate clinical efficacy and safety. However, the combination of nivolumab and ipilimumab resulted in a mere 10% DCR in the TAPUR, leading to an early closure of the cohort.[27] These outcomes underline the need for precision medicine and call for treatments tailored to specific cancer types and drug mechanisms.

Our study is significant for uncovering criteria predictive of ICI treatment. Response and validation of various targeted sequencing panels, including cell-free DNA (cfDNA) derived from patients' blood before treatment. Neither tissue nor blood TMB calculations have been

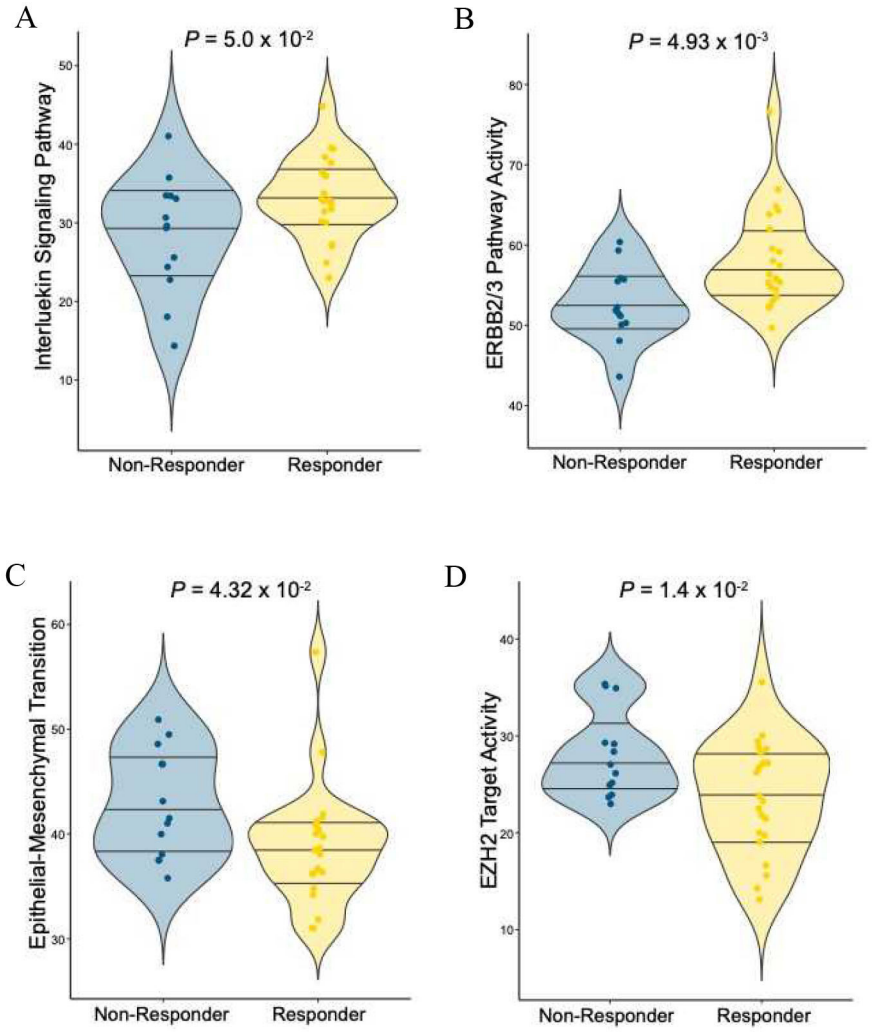

Figure 4 Comparison of interleukin signaling (A), ERBB2/3 pathway (B), epithelial-mesenchymal transition (C), and EZH2 target (D) activities between nivolumab responders and non-responders.

standardized among panels, despite many propositions through global projects.[28] Inferring TMB through cfDNA panel sequencing should be done cautiously due to potential interference from clonal hematopoiesis,[29] particularly considering the relatively low limit of detection of cfDNA genomic profiling. TMB from cfDNA can be up to 2.4 times higher than tissue TMB.[29] In our study, eight patients whose blood had been run through the AXEN liquid panel provided a median TMB of 5 (range 2–14). Five patients presented clinical responses to nivolumab; however, only one had a TMB exceeding 12.

Despite the small sample size, the RNA sequencing data provided significant insights. Patients with a poorer prognosis presented increased expression of epithelial-mesenchymal transition signaling and EZH2 target activity. The increased distribution of interleukin signaling in responders is consistent with previous studies. The notable increase in the ERBB2/3 signal in responders could be interpreted based on our trial population, which had a large proportion of patients with colorectal cancer. ERBB2 expression has been acknowledged as a poor prognostic marker[30] and is associated with reduced T-cell infiltration.[31 32] A recent study reported that ERBB2/3 mutated colorectal cancers are associated with increased MSI-H, TMB-H, and KRAS mutations compared with ERBB2/3 wild-type tumors.[33] Colorectal cancer accounted for 41.7% of our participants; among the responders, more than half (53.8%) were patients with colorectal cancer. Due to the skewed nature of our cohort, the expression of ERBB2/3 might have served as an indicator of MSI-H/TMB-H, potentially leading to good responses with nivolumab. Nevertheless, our data should be interpreted cautiously because of the heterogeneous cohort characteristics and limited sample size for each cancer type.

Extensive research has explored predictive biomarkers related to individuals' immune systems, focusing on the mechanisms of action of ICIs. CD8+T cell count and PD-1 expression are among the investigated biomarkers. To assess systemic immune cell changes, we used PBMC flow cytometry. We evaluated CD8+T cells expressing Ki-67, which indicates active proliferation. The differences in actively proliferating CD8+T cells according to treatment response were more prominent in PD-1- T cells compared

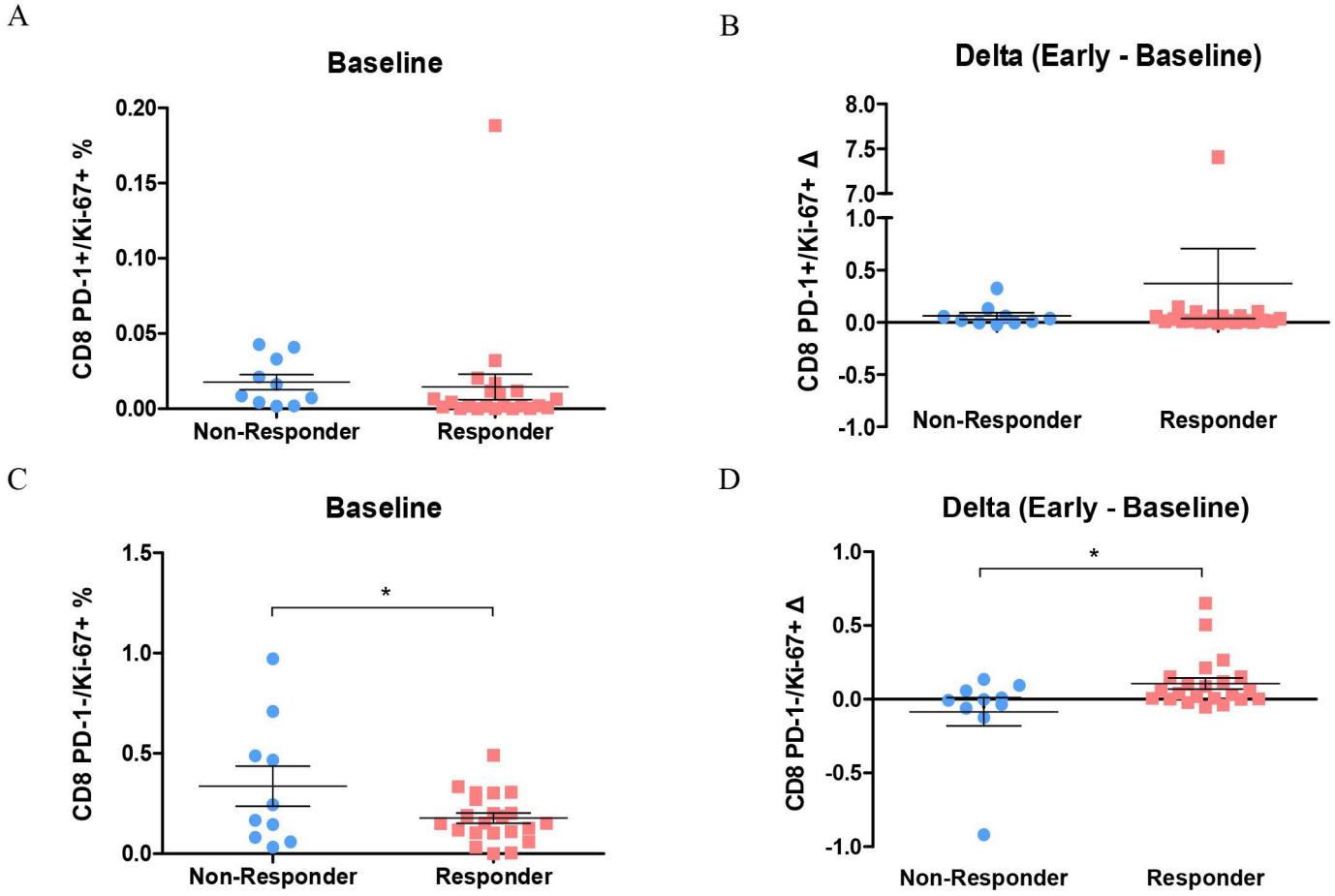

Figure 5 FACS analysis of PBMCs based on treatment response. (A) CD8+/PD-1+/Ki-67+prior to treatment. (B) Alteration in CD8+/PD-1+/Ki-67+from baseline to the early phase (after two cycles) of treatment. (C) CD8+/PD-1-/Ki-67+prior to treatment. (B) Alteration in CD8+/PD-1-/Ki-67+ from baseline to the early phase of treatment.

with PD-1+T cells. Previous studies demonstrated that CD8+/PD-1− cells reflect effector-like and memory-like T cells and are associated with better prognosis.[34] PD-1+T cells are more exhausted than PD-1- cells which express low levels of interferon-gamma and are associated with poorer disease-free survival.[35] In our data, dynamic changes of CD8+/PD-1−/Ki-67+T cells after nivolumab treatment showed more prominent intergroup differences compared with baseline. These findings indicate that patients with active effector T cell proliferation early in treatment, along with high TMB, may exhibit a favorable therapeutic response.

This study has several limitations. First, as a phase 2 clinical trial including various cancer types, the cohort heterogeneity and small sample size resulted in reduced statistical power. The study population included various types of cancers which could have contributed to the low overall response rates of nivolumab single therapy. In addition, we could not thoroughly validate the pathogenicity of variants due to the ethical consideration of granting treatment options to patients with no available therapeutic alternatives. As a result, a few variants with unknown significance were included. Patients with gene mutations indirectly involved in HRD were also included in the enrolment (online supplemental table 1). Such factors could introduce bias into the results. Tumor PD-L1 status was not investigated; therefore, the relationship between TMB and its correlation with treatment response could not be determined.

In conclusion, nivolumab demonstrated moderate efficacy (ORR 17.8%) and manageable toxicity in patients with solid cancer harboring DDR gene mutations. A high TMB (>12 mut/Mb) determined through clinically target sequencing presented significant discriminatory power for the nivolumab response.

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Patient consent for publication Consent obtained directly from patient(s).

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