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ORIGINAL ARTICLE

Efficacy of pembrolizumab in microsatellite-stable, tumor mutational burden-high metastatic colorectal cancer: genomic signatures and clinical outcomes

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Background: Pembrolizumab, an immune checkpoint inhibitor (ICI), shows significant survival benefits in patients with microsatellite instability-high (MSI-H) metastatic colorectal cancer (mCRC), but its efficacy in microsatellite-stable (MSS) mCRC is limited. Although ICIs are effective in tumor mutational burden-high (TMB-H) solid tumors, the impact on MSS-TMB-H mCRC, a rare subset within MSS mCRC, remains unclear.

Materials and methods: We conducted a retrospective analysis using clinical and genomic data from the Center for Cancer Genomics and Advanced Therapeutics (C-CAT) repository in Japan. Patients with MSS-TMB-H mCRC who underwent tissue-based comprehensive genomic profiling and were treated with pembrolizumab or other later-line therapies were included. Pembrolizumab's efficacy was compared with that of trifluridine/tipiracil (FTD/TPI) and regorafenib. Genomic profiles of MSS-TMB-H, MSI-H-TMB-H, and MSS-TMB-low (TMB-L) CRCs were analyzed across 71 cancer-related genes.

Results: Among 127 TMB-H mCRC cases treated with pembrolizumab in the C-CAT repository, 77 were MSS and 50 were MSI-H. Pembrolizumab showed significantly shorter time to treatment failure (TTF) and overall survival (OS) in patients with MSS-TMB-H mCRC compared with those with MSI-H-TMB-H mCRC [median TTF 2.0 versus 10.6 months; hazard ratio (HR) 4.79, 95% confidence interval (CI) 2.65-8.64, median OS 4.5 versus 33.6 months; HR 9.86, 95% CI 3.93-24.77, both $P < 0.0001$]. Among MSS-TMB-H mCRC patients, 19 received pembrolizumab, 73 received FTD/TPI (±bevacizumab), and 18 received regorafenib as their first later-line therapy. Pembrolizumab showed significantly shorter TTF and OS compared with FTD/TPI (median TTF 1.6 versus 4.1 months; HR 2.66, 95% CI 1.41-5.02, $P = 0.0017$, median OS 5.4 versus 13.8 months; HR 2.42, 95% CI, 1.09-5.38, $P = 0.025$). Genomic analysis of 6737 CRCs revealed that MSS-TMB-H CRCs harbored fewer pathogenic alterations than MSI-H-TMB-H CRCs but had a profile similar to MSS-TMB-L CRCs.

Conclusions: Pembrolizumab may be less effective than FTD/TPI in later-line treatment of MSS-TMB-H mCRC, potentially due to genomic similarities between MSS-TMB-H and MSS-TMB-L CRC, suggesting the need for alternative therapeutic strategies in this subgroup.

Key words: colorectal cancer, pembrolizumab, tumor mutational burden, microsatellite instability, comprehensive genomic profiling

INTRODUCTION

The monoclonal antibody pembrolizumab targets programmed cell death protein 1 (PD-1), inhibits its interaction with the programmed death-ligand 1 (PD-L1) and PD-L2 to reactivate the host's antitumor immune response, and demonstrates significant antitumor activity.[1] Immune checkpoint inhibitors (ICIs), including anti-PD-(L)1 and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies, have shown remarkable antitumor effects across various cancer types. Identifying biomarkers useful to select patients likely to benefit from ICI is critical, with microsatellite instability-high (MSI-H) being one of the most well-established, tumor-agnostic biomarkers.[2,3] Additionally,

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pembrolizumab exhibits efficacy in patients with tumor mutational burden-high (TMB-H) solid tumors, defined as having at least 10 mutations per megabase (muts/Mb).[4] These findings led to its approval by the United States Food and Drug Administration in 2021 and in Japan in 2022 for treatment of TMB-H solid tumors.

Nonetheless, ICI efficacy against TMB-H solid tumors varies with cancer type. In particular, ICIs may not improve survival outcomes in patients with microsatellite-stable (MSS) or mismatch repair-proficient (pMMR) tumors. A retrospective study of 1661 pMMR solid tumors from ICI-treated subjects indicated that TMB-H was associated with prolonged survival in non-small-cell lung cancer, melanoma, and head and neck cancers but did not confer a survival benefit in esophageal, gastric, colorectal, or urothelial cancers.[5] Specifically, in subgroup analysis of pMMR colorectal cancer (CRC), no significant differences in overall survival (OS) were observed between TMB $\geq$10 muts/Mb and TMB $<$10 muts/Mb CRCs, except in cases harboring pathogenic mutations in polymerase ε (*POLE*) or polymerase δ1 (*POLD1*) genes.[5] A phase II trial investigating TMB-H (defined as $\geq$9 muts/Mb) and non-MSI-H CRC reported an objective response rate (ORR) of 11% with pembrolizumab therapy.[6] Moreover, one of the three responders in that trial harbored a *POLE* pathogenic mutation. Patients with *POLE/POLD1*-mutated CRC have been reported to demonstrate favorable outcomes with ICIs in retrospective studies.[5,7] These findings overall suggest that anti-PD-1 antibody efficacy against MSS/pMMR TMB-H CRC, excluding cases with *POLE/POLD1* pathogenic mutations, is limited. However, due to the small sample sizes in these studies, it remains difficult to draw definitive conclusions.

The primary treatment strategy for unresectable or metastatic CRC (mCRC) is systemic chemotherapy. For MSI-H mCRC, pembrolizumab monotherapy or combination anti-CTLA-4 antibody/anti-PD-1 antibody therapy has become the standard first- or second-line treatments.[8] In contrast, for MSS mCRC, standard treatment regimens include combinations of fluoropyrimidines, oxaliplatin, irinotecan, and antibodies targeting vascular endothelial growth factor and epidermal growth factor receptor, particularly in *RAS* wild-type cases.[8] In later-line settings, trifluridine/tipiracil (FTD/TPI) (either with or without bevacizumab) or regorafenib monotherapy is recommended, excluding targeted therapies for human epidermal growth factor receptor 2 (HER2)-positive or *BRAF* V600E-positive cases.[8,9] Pembrolizumab therapy has been considered for patients with MSS-TMB-H mCRC in later-line settings, but its comparative efficacy against other later-line treatments remains unclear. Moreover, MSS-TMB-H CRC is a rare subset, accounting for ∼7% of MSS mCRC cases,[10] and TMB can only be assessed quantitatively by next-generation sequencing (NGS)-based testing, making it challenging to prospectively validate pembrolizumab efficacy relative to that of other later-line therapies.

In Japan, NGS-based comprehensive genomic profiling (CGP) testing is covered by the National Health Insurance System and is now part of routine clinical practice. Given the difficulty of conducting prospective studies of rare MSS-TMB-H mCRC, we analyzed real-world clinical and genomic data from a nationwide database in Japan.[11] This study elucidates the real-world efficacy of pembrolizumab therapy and its comparative effectiveness against other later-line therapies in patients with MSS-TMB-H mCRC.

MATERIALS AND METHODS

Study design and patient population

This retrospective study utilized clinical and genomic data from patients with unresectable CRC or mCRC who underwent CGP testing in Japan. CGP results, along with clinical information, were collected by the Center for Cancer Genomics and Advanced Therapeutics (C-CAT). Data were extracted through the C-CAT Research-Use Portal, with all patients providing written informed consent for the secondary use of their clinical information and CGP testing results for research purposes. This study was approved by the C-CAT Data Utilization Review Board (No. CDU2022-036E02) and the Institutional Review Board (IRB) of Kyushu University Hospital (No. 2022-48). This study was conducted between 27 July 2022, the date of approval by both of the IRB of Kyushu University Hospital and the C-CAT for data utilization, and 28 February 2024, the date of data extraction from the C-CAT repository.

Clinical and genomic information obtained from the C-CAT repository

Available clinical data included patient age, sex, histology, and dates of initiation and discontinuation of systemic chemotherapy, as well as the best response and reasons for treatment discontinuation. Treatment response was assessed according to RECIST version 1.1, with evaluations conducted by the treating physicians. The repository provides information on the confirmation dates of disease progression for treatments administered after the submission of CGP testing, enabling the calculation of progression-free survival (PFS). In contrast, for treatments administered before the submission of CGP testing, the repository does not include the confirmation dates of disease progression, making it possible to calculate time to treatment failure (TTF) but not PFS. In Japan, CGP testing is generally conducted during later-line treatments. Among the therapies analyzed in this study, pembrolizumab for MSI-H-TMB-H mCRC and FTD/TPI and regorafenib for MSS-TMB-H mCRC were mostly administered before CGP testing. Consequently, TTF could be calculated for these treatments, but PFS could not be determined. In contrast, pembrolizumab for MSS-TMB-H mCRC is only administered after a CGP test confirms TMB-H status, meaning that all cases of this treatment were initiated post-CGP testing. Therefore, both TTF and PFS could be calculated for pembrolizumab in patients with MSS-TMB-H mCRC. The repository provided detailed information on genomic alterations for all genes

analyzed through CGP testing, although data relevant to alteration copy number were not available.

NGS-based CGP testing

This study included two CGP tests capable of determining both MSI and TMB values: FoundationOne Companion Diagnostic (CDx; Foundation Medicine, Cambridge, MA) and the OncoGuide NCC Oncopanel System (Sysmex Corporation, Hyogo, Japan). Both tests are tumor tissue-based CGP assays designed for formalin-fixed paraffin-embedded tissue specimens. FoundationOne CDx and the NCC Oncopanel assess entire exonic regions of 324 and 124 cancer-related genes, respectively, and can identify four types of genomic alterations: single-nucleotide variants (SNVs), insertions or deletions (indels), copy number alterations, and gene rearrangements, including genomic signatures such as MSI and TMB.[12,13] FoundationOne CDx calculates an MSI score based on the length of repeat sequences in ∼2000 microsatellite regions, classifying results as MSI-H, MS-equivocal, or MSS using criteria established through equivalency testing against the PCR method. The NCC Oncopanel evaluates DNA from both tumor and non-tumor cells from the same patient, calculating an MSI score by comparing the number of homopolymers or microsatellites between tumor-derived and non-tumor-derived DNA. Similar to FoundationOne CDx, the NCC Oncopanel determines MSI-H or MSS status based on PCR-equivalent criteria. TMB scores in FoundationOne CDx are calculated based on the total number of synonymous and non-synonymous mutations with an allele frequency of $\geq$5% per megabase in coding regions, with TMB-H defined as $\geq$10 muts/Mb. In the NCC Oncopanel, TMB is calculated as the number of gene mutations, including SNVs and indels, detected in a target region per megabase, based on the entire length of that sequence, with TMB-H defined as $\geq$10 muts/Mb.

Variant annotation

C-CAT developed a cancer knowledge database (CKDB) containing genomic annotations relevant to specific mutations, categorizing them as oncogenic, inconclusive, of uncertain significance, or likely neutral. In this study, genomic alterations annotated as oncogenic in the CKDB were defined as pathogenic variants. Annotation of *POLE* and *POLD1* mutations was also referenced from the OncoKB database.[14]

Statistical analysis

PFS, TTF, and OS were estimated using the Kaplan—Meier method. Differences between survival curves were evaluated using the log-rank test. PFS was defined as the time from treatment initiation until disease progression or death, TTF was defined as the time from treatment initiation until treatment discontinuation or death, and OS was defined as the time from treatment initiation until death. Patients who were alive, continued treatment, or had missing data at the time of data extraction were considered censored. Survival times were compared according to biomarkers using Cox regression analysis. Categorical variables were analyzed using the chi-square test, and continuous variables were analyzed using the non-parametric Wilcoxon rank sum test. To compare gene alteration frequencies, odds ratios and *P* values were calculated using binomial logistic regression analyses, with *P* values adjusted using the Bonferroni method. *P* values of all reported were two-tailed, and a *P* value of $<$0.05 was considered statistically significant. All statistical analyses were carried out using JMP Pro version 17.0.0 software (SAS Institute Japan, Tokyo, Japan) and EZR version 1.66.[15]

RESULTS

Clinical characteristics and treatment course of TMB-H mCRC patients treated with pembrolizumab

Between June 2019 and February 2024, 127 patients with colorectal or appendiceal adenocarcinoma, characterized by a TMB of $\geq$10 muts/Mb (TMB-H) and MSI status determined through tissue-based CGP testing, who had received pembrolizumab monotherapy, were extracted from the C-CAT repository (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2024.104108). Among them, 77 were MSS (i.e. MSS-TMB-H), and 50 were MSI-H (i.e. MSI-H-TMB-H). The distribution of TMB values among these TMB-H mCRC cases is shown in Supplementary Figure S2A, available at https://doi.org/10.1016/j.esmoop.2024.104108. Cox regression analysis of 127 TMB-H mCRC patients showed consistently longer TTF in the higher TMB subgroup across various TMB cut-offs (Supplementary Figure S2B, available at https://doi.org/10.1016/j.esmoop.2024.104108). However, multivariable analysis identified MSI status, rather than TMB values (using a cut-off of 13 muts/Mb, corresponding to the median TMB value), as an independent predictor of TTF (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2024.104108). Stratified analysis of MSS-TMB-H and MSI-H-TMB-H subgroups revealed no significant correlation between TMB cut-offs and TTF in either group (Supplementary Figure S2C and D, available at https://doi.org/10.1016/j.esmoop.2024.104108).

Table 1 summarizes the clinical characteristics. Patients with MSS-TMB-H mCRC included 34 males (44%) with *KRAS*/*NRAS* mutations in 40 cases (52%), *BRAF* V600E in 7 cases (9%), and *HER2* amplification in 3 cases (4%). The median TMB was 11 muts/Mb. We identified a pathogenic *POLE* P286R mutation in one case showing a remarkably high TMB of 308 muts/Mb. Patients with MSI-H-TMB-H mCRC included 25 males (50%) with *KRAS*/*NRAS* mutations in 14 cases (28%) and *BRAF* V600E in 14 cases (28%). Pembrolizumab was administered at later treatment lines in MSS-TMB-H (median line 4) compared with earlier lines in MSI-H-TMB-H. As detailed in the Materials and Methods, due to data limitations, only TTF and OS could be calculated for MSI-H-TMB-H patients, while TTF, PFS, and OS were calculable for MSS-TMB-H patients. Patients with MSS-TMB-H mCRC demonstrated significantly shorter TTF compared

Table 1. Patient characteristics and clinical outcomes of MSS-TMB-H and MSI-H-TMB-H mCRC patients treated with pembrolizumab

Characteristics	MSS-TMB-H *n* = 77	MSI-H-TMB-H *n* = 50
Age,[a] years, median (range)	65 (36-80)	64 (27-82)
Sex, male, *n* (%)	34 (44)	25 (50)
CGP panel, *n* (%)		
FoundationOne CDx	60 (78)	43 (86)
NCC Oncopanel	17 (22)	7 (14)
KRAS/NRAS, *n* (%)		
Wild type	37 (48)	36 (72)
Mutated (typical[b])	40 (52)	14 (28)
BRAF, *n* (%)		
Wild type	69 (90)	30 (60)
Mutated (V600E)	7 (9)	14 (28)
Mutated (non-V600E)	1 (1)	6 (12)
HER2 amplification, *n* (%)	3 (4)	0
TMB (muts/Mb), *n* (%)		
10.00-14.99	71 (92)	1 (2)
15.00-19.99	5 (6)	0
20.00-99.99	0	39 (78)
≥100.00	1 (1)	10 (20)
Pembrolizumab line, *n* (%)		
First line	0	9 (18)
Second line	5 (6)	23 (46)
Third line	16 (21)	12 (24)
Fourth line	21 (27)	2 (4)
Fifth line	15 (19)	3 (6)
Sixth line or later	20 (26)	1 (2)
Best response, *n* (%)		
Complete response	0	3 (6)
Partial response	1 (1)	10 (20)
Stable disease	7 (9)	19 (38)
Progressive disease	39 (51)	6 (12)
Not evaluable	30 (39)	12 (24)
Reasons for discontinuation, *n* (%)		
Progressive disease	33 (43)	16 (32)
Adverse events	2 (3)	7 (14)
Others	7 (9)	3 (6)
Unknown	6 (8)	3 (6)
Ongoing	29 (38)	21 (42)

CGP, comprehensive genomic profiling; HER2, human epidermal growth factor receptor 2; MSI-H, microsatellite instability-high; MSS, microsatellite-stable; TMB-H, tumor mutational burden-high.
[a]Age at the time of CGP test submission.
[b]Mutations in codons 12, 13, 59, 61, 117, and 146.

with those with MSI-H-TMB-H mCRC [median TTF 2.0 months versus 10.6 months; hazard ratio (HR) 4.79, 95% confidence interval (CI) 2.65-8.64, $P < 0.0001$, Figure 1A]. OS was also significantly shorter in MSS-TMB-H mCRC (median OS 4.5 months versus 33.6 months; HR 9.86, 95% CI 3.93-24.77, $P < 0.0001$, Figure 1B). The median PFS in patients with MSS-TMB-H mCRC was 2.0 months (95% CI 1.6-2.4 months, Figure 1C). The best responses and reasons for treatment discontinuation are shown in Table 1. A substantial proportion of patients were 'not evaluable', including those without imaging evaluations at the time of data extraction or measurable lesions. Response rates were not calculated to avoid bias, but these cases were included in the survival analysis.

TMB-H cases were identified using two CGP tests (FoundationOne CDx and the NCC Oncopanel), with no significant survival differences between the groups (Supplementary Figure S3A and B, available at https://doi.org/10.1016/j.esmoop.2024.104108). Taken together, despite differences in treatment lines, patients with MSS-TMB-H mCRC demonstrated significantly shorter survival following pembrolizumab therapy compared with those with MSI-H-TMB-H mCRC.

Pembrolizumab efficacy versus that of other later-line therapies

To address the comparative efficacy of pembrolizumab versus other therapies in later-line treatment for MSS-TMB-H mCRC, we identified patients with MSS-TMB-H mCRC who had received combinations of fluoropyrimidine, irinotecan, and oxaliplatin as first- or second-line treatments, followed by later-line treatments with either pembrolizumab, regorafenib, or FTD/TPI. These patients were grouped based on the first drug administered in the later-line treatment (Supplementary Figure S1B, available at https://doi.org/10.1016/j.esmoop.2024.104108). To minimize bias, we included only patients who had undergone CGP testing until the fourth line of treatment and who had received at least one of the three anticancer drugs until the fourth line, resulting in 19 patients in the pembrolizumab group, 73 in the FTD/TPI group, and 18 in the regorafenib group (Supplementary Figure S1B, available at https://doi.org/10.1016/j.esmoop.2024.104108). Clinical characteristics for each group are provided in Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2024.104108. The pembrolizumab group included four patients (21%) with detected *BRAF* V600E mutations, a significantly higher proportion compared with the other two groups. In the FTD/TPI group, 55 patients (75%) received bevacizumab along with FTD/TPI. Since most cases in the FTD/TPI and regorafenib groups received these treatments before CGP testing, survival analysis was conducted using TTF. The pembrolizumab group demonstrated significantly shorter TTF compared with the FTD/TPI group (median TTF 1.6 months versus 4.1 months; HR 2.66, 95% CI 1.41-5.02, $P = 0.0017$) (Figure 2A). There was no significant difference in TTF between the pembrolizumab and regorafenib groups (median TTF 1.6 months versus 2.9 months; HR 1.79, 95% CI 0.79-4.07, $P = 0.15$). The median OS was 5.4 months in the pembrolizumab group, 13.8 months in the FTD/TPI group, and 15.0 months in the regorafenib group. OS was significantly longer in the FTD/TPI group compared with the pembrolizumab group (HR 2.42, 95% CI 1.09-5.38, $P = 0.025$, Figure 2B), and there was a trend toward longer OS in the regorafenib group compared with the pembrolizumab group (HR 2.78, 95% CI 0.89-8.66, $P = 0.067$, Figure 2B). The best responses and reasons for treatment discontinuation are shown in Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2024.104108. *BRAF* V600E mutation is associated with poor prognosis in mCRC. Even after excluding cases with *BRAF* V600E, the pembrolizumab group still exhibited significantly shorter TTF and OS compared with the FTD/TPI group (Figure 2C and D). Overall, pembrolizumab demonstrated significantly

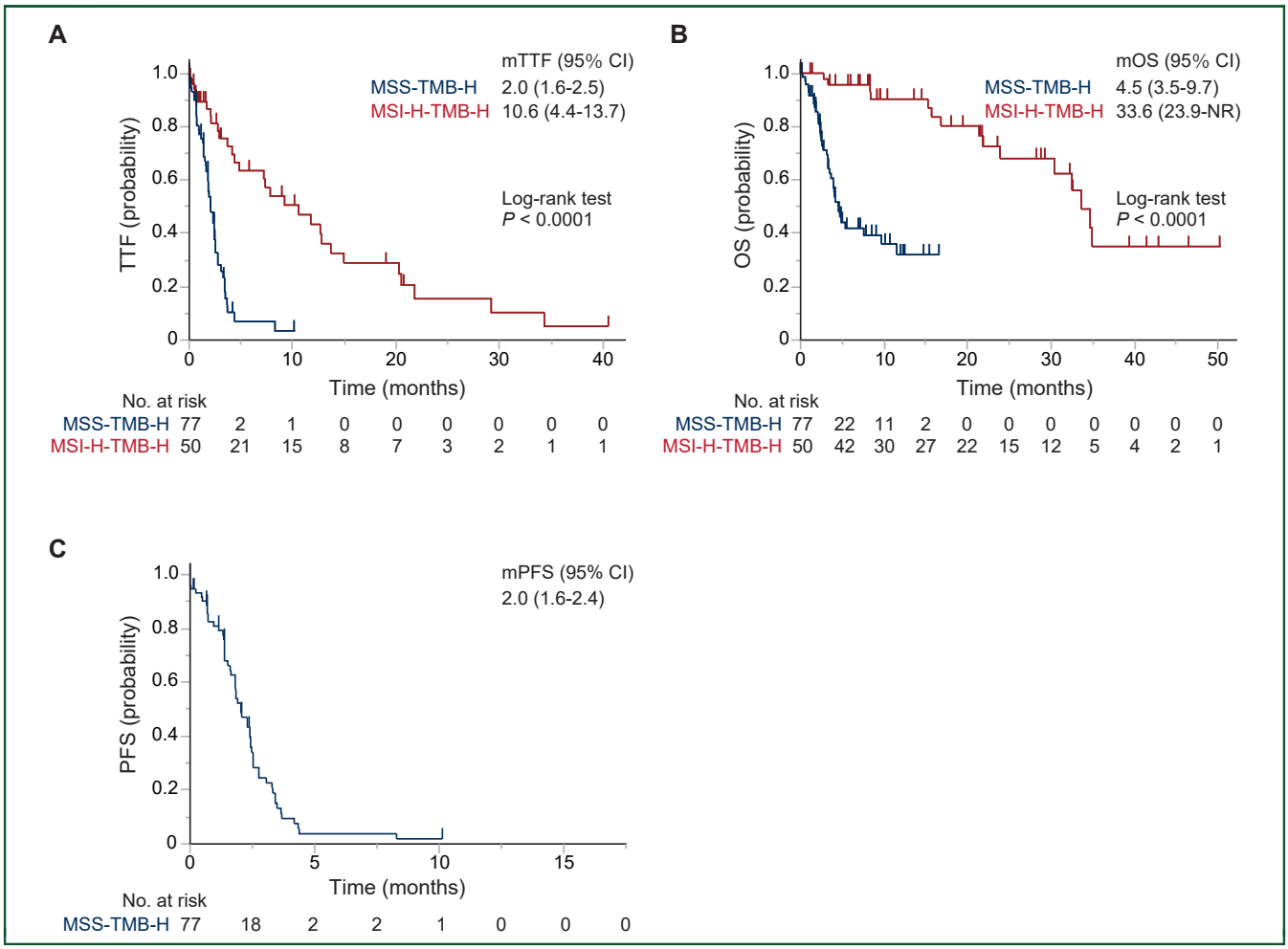

Figure 1. Survival outcomes following pembrolizumab therapy in patients with MSS-TMB-H and MSI-H-TMB-H mCRC. KM curves show TTF (A) and OS (B) in 77 patients with MSS-TMB-H mCRC and 50 patients with MSI-H-TMB-H mCRC. The KM curve shows PFS (C) in 77 patients with MSS-TMB-H mCRC.
CI, confidence interval; CRC, colorectal cancer; KM, Kaplan–Meier; MSI-H, microsatellite instability-high; MSS, microsatellite-stable; mOS, median overall survival; mPFS, median progression-free survival; mTTF, median time to treatment failure; NR, not reached; TMB-H, tumor mutational burden-high.

shorter TTF and OS compared with FTD/TPI in patients with MSS-TMB-H mCRC in the later-line setting.

In the analysis of 110 MSS-TMB-H mCRC patients (Figure 2A), among 86 identified as TMB-H by FoundationOne CDx, the pembrolizumab group demonstrated a significantly shorter TTF compared with the FTD/TPI group and a trend toward shorter OS (Supplementary Figure S3C and D, available at https://doi.org/10.1016/j.esmoop.2024.104108). Similarly, TTF and OS were shorter for pembrolizumab compared with regorafenib. In the 24 patients identified as TMB-H by the NCC Oncopanel, the pembrolizumab group also exhibited trends of shorter TTF and OS compared with the FTD/TPI group, although these differences were not statistically significant (Supplementary Figure S3E and F, available at https://doi.org/10.1016/j.esmoop.2024.104108).

Clinical and genomic features associated with pembrolizumab efficacy

To evaluate the impact of genomic alterations as predictive biomarkers for pembrolizumab efficacy in MSS-TMB-H mCRC, we conducted Cox regression analyses on 77 patients with MSS-TMB-H mCRC treated with pembrolizumab (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2024.104108), examining the presence or absence of genomic alterations with frequencies exceeding 15%, including *BRAF* V600E (Figure 3).

Cox regression analysis revealed that patients harboring *BCL2L1* or *SRC* alterations had significantly longer PFS compared with those without (Figure 3, Supplementary Figure S4A, available at https://doi.org/10.1016/j.esmoop.2024.104108). No other gene alterations were significantly associated with PFS (Figure 3). All pathogenic alterations of *BCL2L1* and *SRC* identified here were gene amplifications. Patients with *BCL2L1* amplification exhibited a trend toward prolonged OS (Supplementary Figure S4B, available at https://doi.org/10.1016/j.esmoop.2024.104108). *BCL2L1* and *SRC*, located on Chr20q11.21 and Chr20q11.23, respectively, were co-amplified in almost all cases (14/16 *BCL2L1*-amplified tumors also had *SRC* amplification). We further investigated the prognostic significance of *BCL2L1* amplification in patients with MSS-TMB-H mCRC treated with FTD/TPI or regorafenib (Supplementary Figure S4C-F,

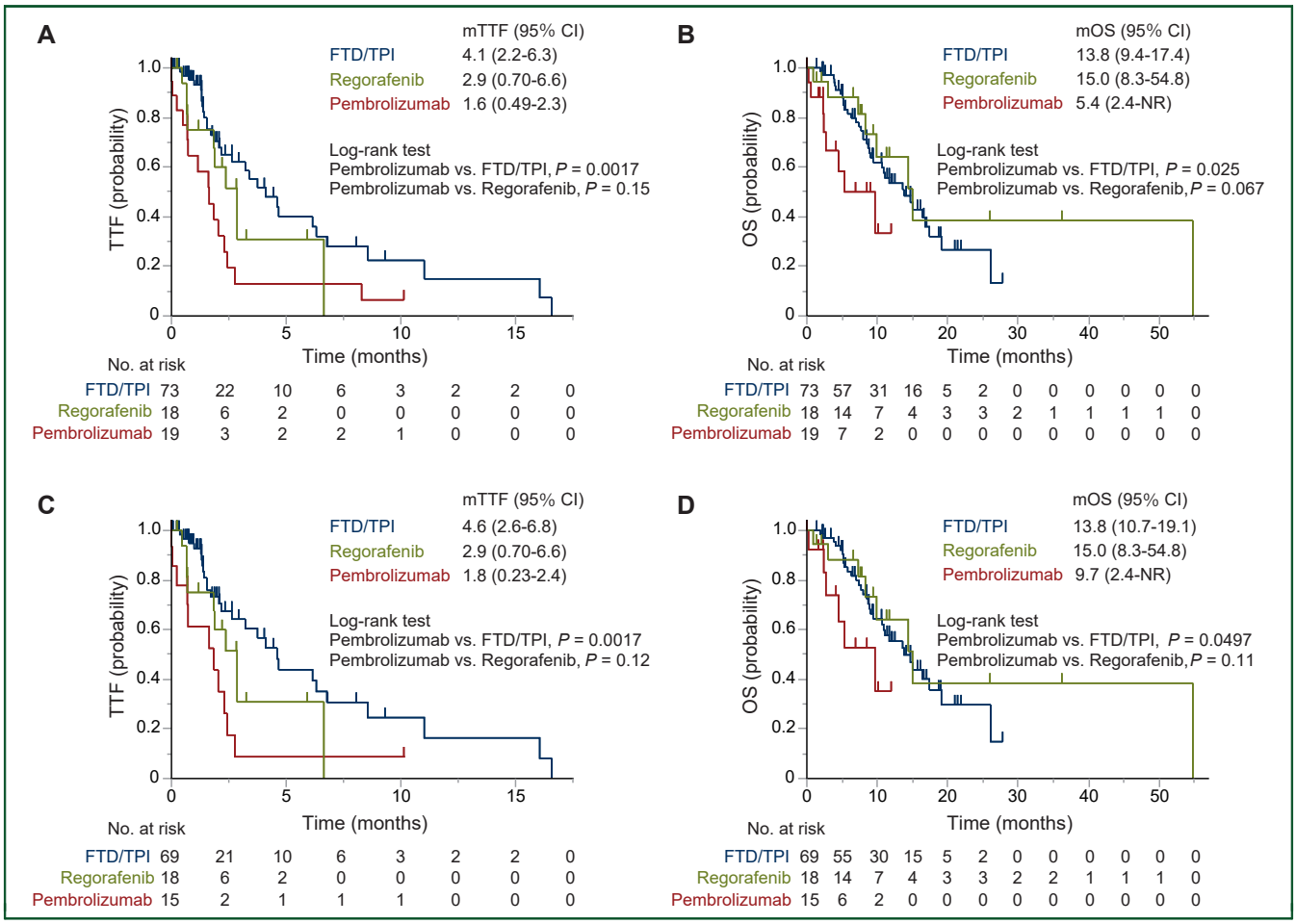

Figure 2. Comparison of survival outcomes among patients with MSS-TMB-H mCRC treated with FTD/TPI, regorafenib, or pembrolizumab in later-line settings. KM curves for TTF (A) and OS (B) in patients treated with FTD/TPI, regorafenib, or pembrolizumab as their first later-line therapy. KM curves for TTF (C) and OS (D) in the same patient groups after excluding cases with the *BRAF* V600E mutation.
FTD/TPI, trifluridine/tipiracil; KM, Kaplan–Meier; NR, not reached; OS, overall survival; TTF, time to treatment failure.

available at https://doi.org/10.1016/j.esmoop.2024.104108). Consistently, *BCL2L1* amplification was associated with longer OS (Supplementary Figure S4D and F, available at https://doi.org/10.1016/j.esmoop.2024.104108). Based on these findings, amplification in the Chr20q11 region may predict better outcomes with pembrolizumab, but it may also suggest a generally favorable prognosis in patients with MSS-TMB-H mCRC.

Distinct and shared genomic profiles of MSI-H-TMB-H, MSS-TMB-H, and MSS-TMB-L CRCs

Among patients with TMB-H mCRC, pembrolizumab efficacy significantly differed between MSI-H-TMB-H and MSS-TMB-H cases (Figure 1, Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2024.104108). Pembrolizumab also did not demonstrate superior efficacy relative to other non-ICI treatments in patients with MSS-TMB-H mCRC (Figure 2). To investigate mechanisms underlying this difference in pembrolizumab sensitivity, we subsequently analyzed the genomic profiles of CRCs. Between June 2019 and February 2024, a total of 6751 patients diagnosed with colorectal or appendiceal adenocarcinoma, with identified TMB value and MSI status through tissue-based CGP testing, were extracted from the C-CAT repository (Supplementary Figure S1C, available at https://doi.org/10.1016/j.esmoop.2024.104108). Among them, 14 cases with goblet cell carcinoid were excluded because this tumor type requires different chemotherapy agents than colorectal adenocarcinoma. The remaining 6737 patients were categorized into four groups based on MSI and TMB status: 6350 (94.3%) were MSS and TMB-low (TMB-L), 310 (4.6%) were MSS and TMB-H, 77 (1.1%) were MSI-H and TMB-H, and none (0%) were MSI-H and TMB-L (Supplementary Figure S1C, available at https://doi.org/10.1016/j.esmoop.2024.104108). The distribution of genomic alterations among the three groups is shown in Figure 4A. TMB values were significantly lower in MSS-TMB-H CRC compared with MSI-H-TMB-H CRC (median 11 muts/Mb versus 52 muts/Mb, $P < 0.0001$, Figure 4B). Among MSS-TMB-H CRCs, 295 cases (95%) had TMB values <20 muts/Mb, while only 8 cases (2.6%) had TMB >100 muts/Mb. Pathogenic variants of *POLE* referenced from OncoKB[14] were detected in all eight of these hypermutated cases except one. We subsequently compared the frequency

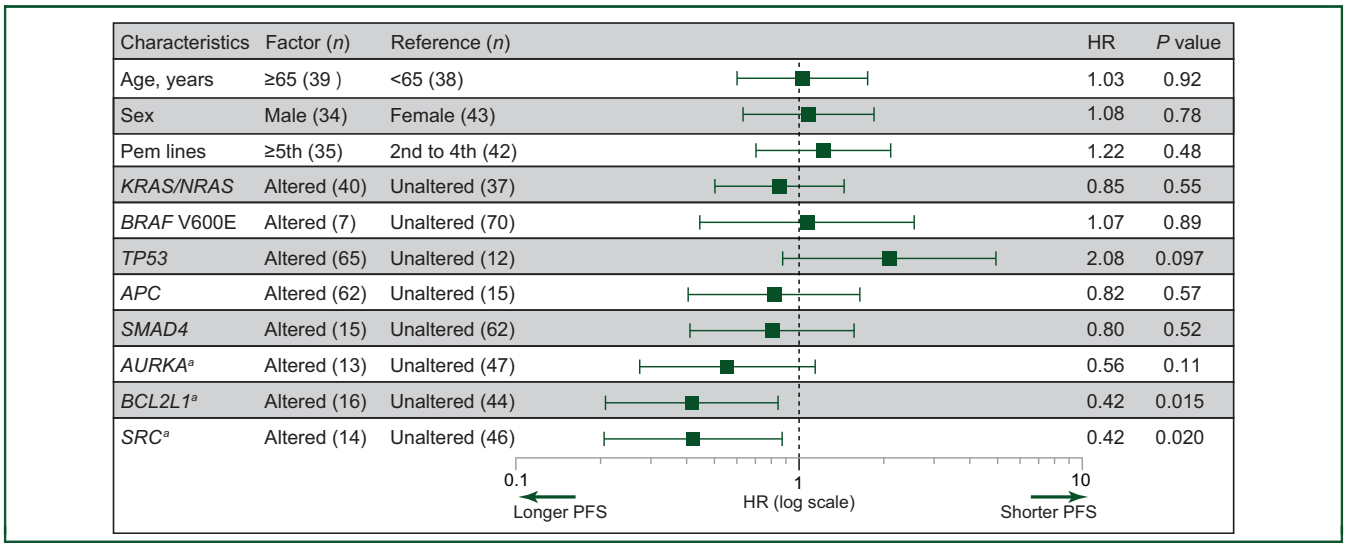

Figure 3. Univariate Cox regression analyses of PFS following pembrolizumab therapy in 77 patients with MSS-TMB-H mCRC. *P* values were calculated using the Wald test. The dashed vertical line indicates an HR of 1. Boxes and whiskers represent median and 95% CI, respectively.
CI, confidence interval; HR, hazard ratio; mCRC, metastatic colorectal cancer; Pem, pembrolizumab; PFS, progression-free survival; MSS, microsatellite-stable; TMB-H, tumor mutational burden-high.
[a]Note that 17 patients were excluded from this analysis due to the absence of gene assessment by the NCC Oncopanel.

of pathogenic alterations among the three groups for the 71 genes that were commonly analyzed by both FoundationOne CDx and the NCC Oncopanel, and for which pathogenic alterations were detected in at least 3% of cases in one or more of the three groups. The list of the 71 genes and their frequencies of pathogenic alterations is shown in Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2024.104108. Comparing MSS-TMB-H with MSI-H-TMB-H, 38 of 71 genes (54%) were more frequently altered in MSI-H-TMB-H (Figure 4C). In contrast, only *TP53*, *APC*, and *KRAS* were frequently altered in MSS-TMB-H (Figure 4C). Comparing MSS-TMB-H with MSS-TMB-L, genes such as *POLE*, *MSH2*, *BRCA1*, and *PBRM1* were more frequently altered in MSS-TMB-H (Figure 4D). Only 10 of 71 genes (14%) showed significant frequency differences, far fewer than in MSS-TMB-H versus MSI-H-TMB-H (Figure 4E). Hypermutated CRCs with pathogenic *POLE* mutations are considered a distinct subtype among MSS-TMB-H CRC.[16] Similar analyses excluding seven pathogenic *POLE*-mutated cases yielded consistent findings, with 33 of 70 genes (47%) showing significant alteration-rate differences between MSS-TMB-H and MSI-H-TMB-H (Figure 4F and H), but we observed no significant differences in alteration frequency between MSS-TMB-H and MSS-TMB-L (0/70 genes; Figure 4G and H). Overall, MSS-TMB-H CRC exhibited significantly lower TMB values and fewer pathogenic alterations compared with MSI-H-TMB-H CRC. MSS-TMB-H and MSS-TMB-L CRCs displayed similar genomic profiles, particularly when pathogenic *POLE*-mutant cases were excluded.

DISCUSSION

MSI-H or MMR-deficient (dMMR) is a well-established predictive marker for ICI efficacy in mCRC. TMB of ≥10 muts/Mb has also been suggested to serve as a favorable factor for ICI efficacy in mCRC.[17-19] However, many studies do not distinguish MSI-H/dMMR from MSS/pMMR cases, potentially overestimating the predictive value of 'TMB ≥ 10' due to inclusion of MSI-H cases with inherently better outcomes. Our findings reveal that despite differences in treatment lines, pembrolizumab was notably less effective in patients with MSS-TMB-H mCRC than in those with MSI-H-TMB-H mCRC. This observation aligns with a meta-analysis that reported only 8.5% ORR to ICI-based regimens in pMMR/non-MSI-H mCRC, highlighting the limited efficacy of ICI-based therapy in this subset.[20] Moreover, survival outcomes did not differ significantly between pMMR mCRC cases with TMB ≥10 muts/Mb and those with TMB <10 muts/Mb after ICI treatment.[5] Although ICIs are not approved for use in MSS mCRC with TMB <10 muts/Mb in Japan, making direct comparisons impossible, our results suggest that MSS-TMB-H mCRC may exhibit reduced sensitivity to ICIs compared with MSI-H-TMB-H mCRC, indicating that specific TMB cut-offs may not reliably predict ICI efficacy in MSS/pMMR mCRC.

We also found that pembrolizumab is associated with significantly shorter TTF and OS compared with FTD/TPI (±bevacizumab) in later-line treatment of patients with MSS-TMB-H mCRC. Due to data limitations, PFS could not be estimated in this study and thus we used TTF as an alternative endpoint. TTF is increasingly recognized as a practical surrogate for PFS in real-world data.[21,22] Actually, the median TTF for FTD/TPI (±bevacizumab) therapy observed in this study was consistent with the median PFS ranging from 2.0 to 5.6 months reported in previous prospective trials.[23,24] Therefore, our results suggest that pembrolizumab may be less effective than FTD/TPI in later-line treatment of MSS-TMB-H mCRC. On the other hand, the median OS in the FTD/TPI and regorafenib groups in this study was longer than previously reported in prospective trials ranging from 6.4 to 10.8 months.[23-25] This discrepancy may be attributed to the characteristics of patients eligible for CGP testing under

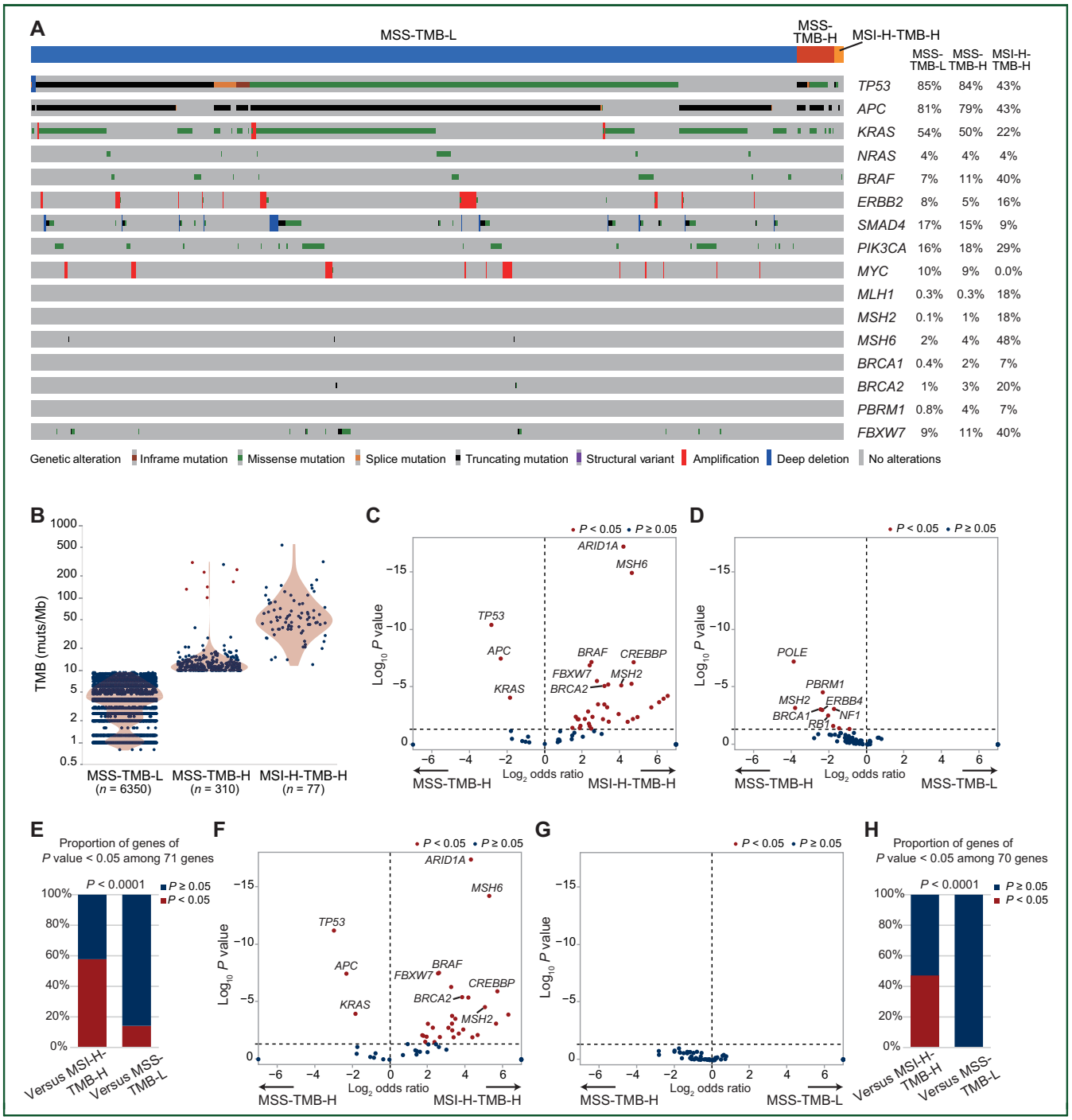

Figure 4. Comprehensive analysis of genomic features in 6737 CRC cases. (A) OncoPrint representation of mutations, copy number alterations, and translocations in key driver genes across MSS-TMB-L, MSS-TMB-H, and MSI-H-TMB-H CRCs, generated via cBioPortal.[34,35] (B) TMB distribution across the three groups stratified by TMB and MSI status. Red dots represent the cases with *POLE* pathogenic mutations. (C, D) Volcano plots showing differential gene alteration analysis of 71 genes in MSI-H-TMB-H CRC relative to MSS-TMB-H CRC (C), and in MSS-TMB-L CRC relative to MSS-TMB-H CRC (D). (E) Bar plots comparing frequencies of significantly altered genes ($P < 0.05$) among the 71 genes analyzed in Figure 4C and D. (F, G) Volcano plots of differential gene alteration analysis (excluding *POLE* mutations) of 70 genes in MSI-H-TMB-H CRC relative to MSS-TMB-H CRC (F), and in MSS-TMB-L CRC relative to MSS-TMB-H CRC (G). Seven patients with *POLE* pathogenic mutations were excluded. (H) Bar plots comparing frequencies of significantly altered genes ($P < 0.05$) among the 70 genes analyzed in Figure 4F and G.
CRC, colorectal cancer; MSI-H, microsatellite instability-high; MSS, microsatellite-stable; TMB-H, tumor mutational burden-high; TMB-L, TMB-low.

Japan's National Health Insurance System, which restricts CGP testing to patients with a certain expected survival prognosis. As a result, many of the patients included in this study were likely those with preserved overall condition who were deemed suitable for CGP testing during later-line treatments (e.g. third- or fourth-line therapy).

Several factors may underlie the limited efficacy of ICI seen in patients with MSS-TMB-H mCRC. The predictive value of TMB for ICI efficacy varies across cancer types, with some cancers showing high sensitivity and specificity for TMB-H, and others not.[26,27] This variability may stem from differential effects of TMB on the tumor immune

microenvironment. TMB and neoantigen load of the tumor are reportedly highly correlated.[27] In cancers in which CD8+ T-cell infiltration levels positively correlate with neoantigen load, such as melanoma, lung, and bladder cancers, TMB-H tumors show greater responses to ICI compared with TMB-L tumors.[27] Conversely, in cancers like breast and prostate, in which CD8+ T-cell infiltration does not correlate with neoantigen load, TMB-H tumors exhibit lower response rates to ICIs compared with TMB-L tumors.[27] Moreover, specific genetic alterations may influence ICI sensitivity. Comprehensive genomic analyses of MSS gastrointestinal cancers have shown that mutations in genes like *SMAD2*, *MTOR*, and *KEAP1* are linked to favorable ICI responses and higher expression of interferon-γ signature genes.[28] In contrast, mutations in *AKT1* and *CDH1* in MSI-H/dMMR gastrointestinal cancers have been identified as independent predictors of ICI resistance.[29] Mutations in genes of the NRF2 pathway, which are observed in lung squamous cell carcinoma, also reportedly negatively impact PFS and decreased the predictive accuracy of PD-L1 and TMB status, further complicating treatment strategies.[30] Moreover, mutations in genes encoding components of the SWI/SNF chromatin remodeling complex, which are frequently seen in TMB-H tumors, are associated with favorable ICI efficacy across several cancers, including CRC.[31] These findings underscore the complex interplay between specific genetic alterations and the immune microenvironment in determining ICI efficacy.

This study reveals that MSS-TMB-H CRC exhibits significantly lower TMB values and fewer pathogenic alterations relative to MSI-H-TMB-H CRC. If specific genomic changes do in fact influence ICI efficacy, MSI-H-TMB-H CRC likely accumulates more alterations in key genes over time than does MSS-TMB-H CRC, enhancing sensitivity of the former to ICIs. In contrast, we observed no significant difference in the frequency of pathogenic alterations between MSS-TMB-L CRC and MSS-TMB-H CRC, excluding cases with *POLE* pathogenic mutations. This genomic similarity may explain why MSS-TMB-H CRC exhibits ICI sensitivity similar to that of MSS-TMB-L CRC.

Analysis presented here also suggests that patients with MSS-TMB-H mCRC harboring Chr20q11 amplification exhibit longer PFS and OS after pembrolizumab therapy than do those lacking this amplification. Tumors with high infiltration of immune cells, such as CD8+ T cells ('immune hot' tumors), typically respond well to ICIs, whereas 'immune cold' tumors with low immune cell infiltration generally show limited efficacy.[32] Although a previous study classified CRCs with Chr20q11 amplification as immune cold,[33] suggestive of a poor ICI response, we observed the opposite trend. Cases with *BCL2L1* amplification also demonstrated longer OS, even following treatment with non-ICI therapies like FTD/TPI and regorafenib. This, along with previous finding that Chr20q11-amplified CRCs are associated with favorable prognosis,[33] suggests that these tumors represent a subgroup with better overall outcomes and could account for longer PFS seen after pembrolizumab therapy.

This study has limitations, including its retrospective design, limited sample size, and reliance on registry data, which lacks the robustness of prospective studies and insights into treatment selection rationale. Pembrolizumab may have been chosen for patients with frailer performance status due to its perceived favorable side-effect profile, potentially introducing bias in treatment outcomes. Additionally, in Japan, CGP testing is restricted to patients with adequate prognosis, excluding those with rapid disease progression and very poor prognosis. Moreover, differences in TMB measurement methods (FoundationOne and NCC Oncopanel) create complications; while FoundationOne results aligned with overall findings, the small NCC Oncopanel sample size limits conclusions. Therefore, these findings should be further validated in larger-scale studies or prospective trials using TMB-H as a selection criterion.

Conclusions

Our findings provide real-world evidence of pembrolizumab efficacy in a rare subset of MSS-TMB-H mCRC. In later-line treatment, pembrolizumab appears less effective than FTD/TPI for patients with MSS-TMB-H mCRC, likely because the MSS-TMB-H mCRC genomic signature differs from that of MSI-H-TMB-H CRC and more closely resembles that of MSS-TMB-L CRC.

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