Mutational analysis of microsatellite-stable gastrointestinal cancer with high tumour mutational burden: a retrospective cohort study

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Summary

Background Genomic signatures contributing to high tumour mutational burden (TMB-H) independent from mismatch-repair deficiency (dMMR) or microsatellite instability-high (MSI-H) status are not well studied. We aimed to characterise molecular features of microsatellite stable (MSS) TMB-H gastrointestinal tumours.

Methods Molecular alterations of 48 606 gastrointestinal tumours from Caris Life Sciences (CARIS) identified with next-generation sequencing were compared among MSS–TMB-H, dMMR/MSI-H, and MSS–TMB-low (L) tumours, using χ^2 or Fisher's exact tests. Antitumour immune response within the tumour environment was predicted by analysing the infiltration of immune cells and immune signatures using The Cancer Genome Atlas database. The Kaplan-Meier method and the log-rank test were used to evaluate the impact of gene alterations on the efficacy of immune checkpoint inhibitors in MSS gastrointestinal cancers from the CARIS database, a Memorial Sloan Kettering Cancer Center cohort, and a Peking University Cancer Hospital cohort.

Findings MSS–TMB-H was observed in 1600 (3·29%) of 48 606 tumours, dMMR/MSI-H in 2272 (4·67%), and MSS–TMB-L in 44 734 (92·03%). Gene mutations in *SMAD2, MTOR, NFE2L2, RB1, KEAP1, TERT,* and *RASA1* might impair antitumour immune response despite TMB-H, while mutations in 16 other genes (*CDC73*, *CTNNA1*, *ERBB4*, *EZH2*, *JAK2*, *MAP2K1*, *MAP2K4*, *PIK3R1*, *POLE*, *PPP2R1A*, *PPP2R2A*, *PTPN11*, *RAF1*, *RUNX1*, *STAG2*, and *XPO1*) were related to TMB-H with enhanced antitumour immune response independent of dMMR/MSI-H, constructing a predictive model (modified TMB [mTMB]) for immune checkpoint inhibitor efficacy. Patients with any mutation in the mTMB gene signature, in comparison with patients with mTMB wildtype tumours, showed a superior survival benefit from immune checkpoint inhibitors in MSS gastrointestinal cancers in the CARIS cohort (n=95, median overall survival 18·77 months [95% CI 17·30–20·23] *vs* 7·03 months [5·73–8·34]; hazard ratio 0·55 [95% CI 0·31–0·99], p=0·044). In addition, copy number amplification in chromosome 11q13 (eg, *CCND1*, FGF genes) was more prevalent in MSS–TMB-H tumours than in the dMMR/MSI-H or MSS–TMB-L subgroups.

Interpretation Not all mutations related to TMB-H can enhance antitumour immune response. More composite biomarkers should be investigated (eg, mTMB signature) to tailor treatment with immune checkpoint inhibitors. Our data also provide novel insights for the combination of immune checkpoint inhibitors and drugs targeting cyclin D1 or FGFs.

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Introduction

The introduction of immune checkpoint inhibitors targeting PD-1 or PD-L1 has revolutionised cancer therapy, providing robust and durable responses in a subset of patients with some types of cancer. The variability of response to immune checkpoint inhibitors highlights the unmet need for identifying and validating predictive biomarkers for immune checkpoint inhibitor efficacy. As a proxy for the expression of tumour-specific neoantigens, high tumour mutational burden (TMB-H) was reported to be associated with durable responses on the basis of the KEYNOTE-158 trial, accelerating the pan-cancer approval by the US Food and Drug Administration (FDA) of pembrolizumab to treat patients with TMB-H (≥10 mutations per megabase) advanced solid tumours.[1] However, KEYNOTE-158 might not be representative across tumour types, as the clinical benefit varied widely by tumour histology and

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Research in context

Evidence before this study

We searched PubMed for peer-reviewed, original studies (with no start date and up to Jan 10, 2022), using the search terms "tumour mutation burden", "immune checkpoint inhibitor", "microsatellite stable", and "cancer". We also reviewed congress abstracts in the field of immunotherapy in microsatellite stable (MSS) cancers. Although pembrolizumab is recommended for patients with high tumour mutational burden (TMB-H) solid tumours on the basis of the KEYNOTE-158 trial, studies of MSS gastrointestinal cancers have shown mixed results for TMB-H as a predictive biomarker for efficacy of immune checkpoint inhibitors. Increasing evidence suggests that quality of neoantigens, which could be affected by genetic mutations, might predict immune checkpoint inhibitor efficacy more accurately than the quantity. Several gene mutations, such as *POLE*, are associated with TMB, independent of microsatellite instability-high (MSI-H) status. However, no systematic analysis of molecular features of MSS gastrointestinal tumours with TMB-H has been reported.

Added value of this study

This is the largest study to our knowledge to explore the molecular features of MSS gastrointestinal tumours with TMB-H, providing novel insights for patient selection, the biology of the tumour–immune interaction, and the development of rational immunotherapy combinations in the future. Mutations in *SMAD2*, *MTOR*, *NFE2L2*, *RB1*, *KEAP1*, *TERT*, and *RASA1* were associated with suppressed tumour immune environment, despite high TMB. Gene mutations in 16 genes (*CDC73*, *CTNNA1*, *ERBB4*, *EZH2*, *JAK2*, *MAP2K1*, *MAP2K4*, *PIK3R1*, *POLE*, *PPP2R1A*, *PPP2R2A*, *PTPN11*, *RAF1*, *RUNX1*, *STAG2*, and *XPO1*) were related to TMB-H with improved antitumour immune response independent of mismatch-repair deficiency or MSI-H; we used these genes to construct a predictive model (modified TMB [mTMB]) for immune checkpoint inhibitor efficacy. Copy number amplification in chromosome 11q13 (*CCND1*, FGF genes) was more prevalent in MSS–TMB-H tumours than in the MMS–TMB-L and dMMR/MSI-H subgroups.

Implications of all the available evidence

Our study indicated that not all the gene mutations associated with TMB-H can improve antitumour immune response. Mutations in *SMAD2*, *MTOR*, *NFE2L2*, *RB1*, *KEAP1*, *TERT*, and *RASA1* should be taken into consideration when patients with TMB-H are considered for immune checkpoint inhibitor treatment. In addition to TMB, more composite biomarkers should be developed (such as the mTMB signature) for more effective patient selection for treatment with immune checkpoint inhibitors. Our data also provide novel insights for the combination of immune checkpoint inhibitors and drugs targeting cyclin D1 or FGFs.

some common cancer types (such as colorectal cancer) were not included in the prospective biomarker analysis of the trial. Furthermore, assessment was limited to overall response rate rather than survival advantage. These concerns limit the generalisability of TMB as a robust tissue-agnostic marker.

Microsatellite instability-high (MSI-H) status is well recognised as a positive predictor for immune checkpoint inhibitor efficacy; however, more than 90% of gastrointestinal tumours are microsatellite stable (MSS). Studies in patients with MSS gastrointestinal cancers have shown mixed results for TMB-H as a predictive biomarker for immune checkpoint inhibitor efficacy, including use of the FDA-approved threshold of 10 mutations per megabase. Xu and colleagues observed a higher overall response rate in TMB-H gastric cancer than in TMB-low (L) gastric cancer, without consideration of mismatch-repair deficiency (dMMR) or MSI-H status.[2] However, a retrospective analysis of 251 gastrointestinal tumours (derived from a database of 1678 MSS solid tumours) from Memorial Sloan Kettering Cancer Center (MSKCC; New York, NY, USA) revealed no significant association between TMB-H and clinical benefit (including overall response rate, progression-free survival, and overall survival).[3] Furthermore, a study by Kim and colleagues,[4] the EPOC1603 study,[5] and a study by Marabelle and colleagues[1] did not observe a significant association between TMB and overall response rate in MSS gastric cancer, colorectal cancer, or anal cancer, respectively. These data suggest caution in using TMB as a predictive biomarker for immune checkpoint inhibitor efficacy in MSS gastrointestinal tumours despite the FDA tumour-agnostic approval of pembrolizumab.

Increasing evidence suggests that the quality of neoantigens might predict immune checkpoint inhibitor efficacy more accurately than the quantity. Genetic mutations can have an impact on peptide presentation, intratumoural heterogeneity, and the tumour immune microenvironment, all of which further influence the immunogenicity of neoantigens.[6] However, this complex biological process is not accounted for by the current TMB scoring system. Thus, improved understanding of molecular features of MSS gastrointestinal tumours with TMB-H could provide novel insights for patient selection, the biology of the tumour–immune interaction, and the development of more rational immunotherapy combinations in the future. In this study, we aimed to explore the impact of molecular features associated with TMB-H on the tumour immune microenvironment and the survival benefit of immune checkpoint inhibitor treatment in MSS gastrointestinal tumours.

Methods

Tumour samples

A total of 48 606 samples with pathologically confirmed gastrointestinal cancer were collected in the

USA by a commercial Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory (Caris Life Sciences, Phoenix, AZ, USA; referred to as CARIS). Formalin-fixed paraffin-embedded tissue blocks were prepared at each patient's submitting pathology department according to each facility's own standard protocols. The tissue blocks were cut into 4 µm sections using microtomes. All the samples with known TMB and microsatellite stability status were included in our study. International cases and tumours of non-gastrointestinal cancer types were excluded. In addition, cases that were tested for TMB and microsatellite stability status but yielded no informative results (ie, the results were indeterminate due to low coverage for the particular gene) were excluded from the denominators when calculating the prevalence of a particular mutation. All tests associated with this study have met the requirements of CLIA, the College of American Pathologists, and the International Organization for Standardization.[7,8] In addition, clinical data and tissue were collected from public databases (Peking University Cancer Hospital [PUCH] in Beijing, China, and MSKCC) for immune checkpoint inhibitor-related survival analyses and from The Cancer Genome Atlas (TCGA) for both non-immune checkpoint inhibitor-related survival analyses and immune landscape analyses (appendix p 16). Study design is shown in the appendix (p 1).

Our study was conducted according to institutional review board guidelines. Since this is a retrospective biomarker study with all data deidentified, our study is considered exempt from institutional review board approval (sponsor protocol number: CCC-001-0320).

The collections of clinical data and tissues were approved by TCGA-specific institutional review boards,[9] MSKCC,[10] and PUCH.[11]

Genetic analysis

Next-generation sequencing was performed on genomic DNA isolated from formalin-fixed paraffin embedded tumour samples from the CARIS cohort using the NextSeq or NovaSeq 6000 platforms (Illumina, San Diego, CA, USA). For NextSeq-sequenced tumours, a custom-designed SureSelect XT assay (Agilent Technologies, Santa Clara, CA, USA) was used to enrich 592 whole-gene targets. For NovaSeq-sequenced tumours, more than 700 clinically relevant genes at high coverage and high read-depth (>700×) were used, along with another panel designed to enrich for more than 20 000 additional genes at lower depth (>200×). All variants were detected with more than 99% confidence based on allele frequency and amplicon coverage, with an average sequencing depth of coverage of more than 500× and an analytic sensitivity of 5%. Before molecular testing, tumour enrichment was achieved by harvesting targeted tissue using manual microdissection techniques. Genetic variants identified were interpreted by board-certified molecular geneticists (author MO is the leader of pathologists and geneticists from CARIS) and were categorised as "pathogenic", "likely pathogenic", "variant of unknown significance", "likely benign", or "benign", according to the American College of Medical Genetics and Genomics standards. When assessing mutation frequencies of individual genes, "pathogenic", and "likely pathogenic" were counted as mutations. The copy number alteration of each exon was determined by calculating the average depth of the sample along with the sequencing depth of each exon and comparing this calculated result with a precalibrated value. TMB-H was defined as ten or more mutations per megabase, according to the Friends of Cancer Research TMB Harmonization Project.[12] Genomic features were also compared when 20 or more and 50 or more mutations per megabase were used as the cutoff for TMB-H.

A combination of multiple test platforms was used to determine the MSI or MMR status, including fragment analysis (MSI Analysis System kit; Promega, Madison, WI, USA), immunohistochemistry staining (MLH1, M1 antibody; MSH2, G2191129 antibody; MSH6, 44 antibody; and PMS2, EPR3947 antibody; Ventana Medical Systems, Tucson, AZ, USA), and next-generation sequencing (7000 target microsatellite loci were examined and compared with the reference genome hg19 from the University of California) in the CARIS cohort. The three platforms generated highly concordant results, and in the rare cases of discordant results, the microsatellite stability or MMR status of the tumour was determined in the order of immunohistochemistry, fragment analysis, and next-generation sequencing. Tumour samples were classified into three groups: MSS–TMB-H, dMMR/MSI-H, and MSS–TMB-L. All analyses were done in each group. More details on fusion detection, immunohistochemistry staining, and chromogenic in situ hybridisation (CISH) are described in the appendix (pp 16–17).

See Online for appendix

Establishment of a modified TMB signature

dMMR/MSI-H and higher TMB were reported to be associated with better response to immune checkpoint inhibitors.[13,14] Therefore, genes with exclusively highest mutation rates in the MSS–TMB-H subgroup (referred to as gene panel A) and genes with similar mutation rates in the dMMR/MSI-H and MSS–TMB-H subgroups, but significantly lower in the MSS–TMB-L subgroup (referred to as gene panel B) were explored for the potential association with antitumour immune response using the immune signature scores (calculated by averaging the expression value of included genes in the corresponding signature gene sets) and the infiltration of immune cells (estimated using CIBERSORT and xCell algorithms; appendix pp 17–18) in the TCGA cohort. Only genes with a positive association with antitumour immune response in MSS gastrointestinal tumours were further used to construct the predictive model for immune checkpoint inhibitor treatment. We refer to these genes collectively as

the modified TMB (mTMB) gene signature. We explored the impact of the mTMB gene signature on the tumour immune microenvironment and the efficacy of immune checkpoint inhibitor in the MSS gastrointestinal tumour cohorts treated with immune checkpoint inhibitors.

Survival analysis

95 patients in the CARIS database who received pembrolizumab or nivolumab according to local or national treatment guidelines between June 1, 2016, and April 30, 2021) were analysed retrospectively for overall survival. 192 patients in the MSKCC database and 37 patients in the PUCH database treated with immune checkpoint inhibitors were also analysed for overall survival. 1574 patients in the TCGA database who were not treated with immune checkpoint inhibitors (but were treated with chemotherapy or surgery) were analysed for overall survival. Immune-related overall survival was defined as the time from initial immunotherapy treatment to the day of death or the end of follow-up. Overall survival in the TCGA cohort was defined as the time from initial diagnosis or the start of treatment to death.

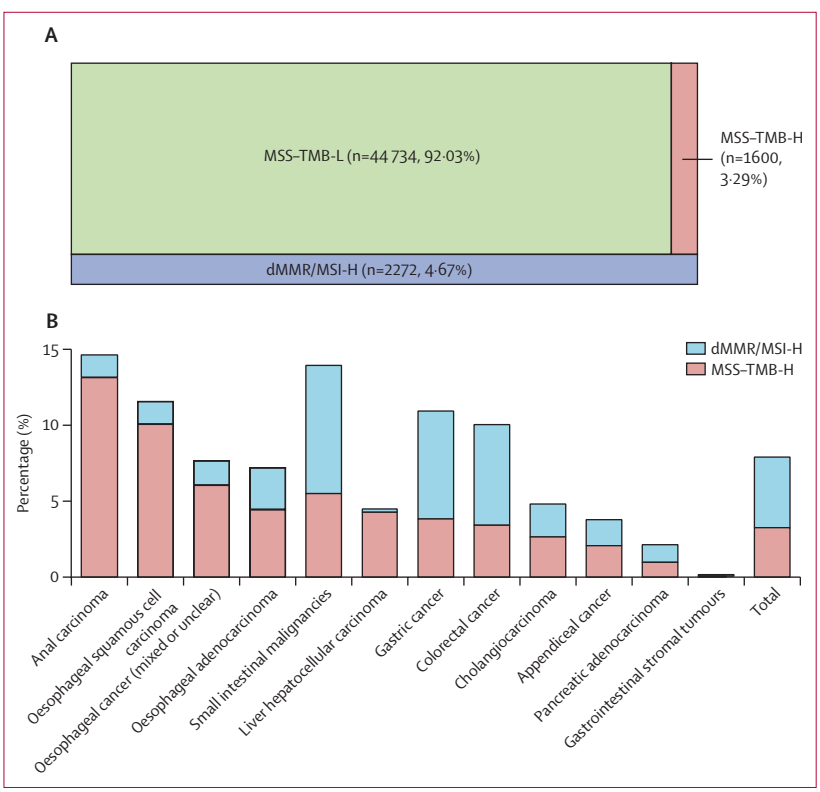

Figure 1: **The relationship between dMMR/MSI-H and TMB-H status in gastrointestinal cancers in the CARIS cohort**

(A) An overview of the distribution of MSI and TMB status. Of 48 606 total tumours, 1600 were MSS–TMB-H, 2272 were dMMR/MSI-H, and 44 734 were MSS–TMB-L. The TMB-H cutoff was 10 or more mutations per megabase. (B) Distribution of MSI and TMB status among various tumour histologies (n=12) in gastrointestinal cancers. dMMR=mismatch-repair deficiency. MSI-H=microsatellite instability-high. MSS=microsatellite stable. TMB-H/L=tumour mutational burden-high/low.

Statistical analysis

Statistical analysis was performed with R (version 3.5.0) and SPSS (version 26.0). Continuous data were compared using the Mann-Whitney *U* test. Categorical data were evaluated using Fisher's exact test or the χ^2 test, with the false-discovery rate (q value) controlled to 0·05 using the Benjamini-Hochberg procedure for multiple and pairwise comparison. We used the Kaplan-Meier method to estimate survival functions and the log-rank test to compare survival distributions. In a sensitivity analysis, we included immune checkpoint inhibitor-treated patients ranked in the top 95% of longest to shortest follow-up time in the CARIS cohort. All p values less than 0·05 were considered significant.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

The most common tumour type in the CARIS cohort was colorectal cancer (appendix p 2). The overall prevalence of MSS–TMB-H in the whole cohort was 3·29% (1600 of 48 606 tumours), while dMMR/MSI-H and MSS–TMB-L were observed in 4·67% (2272 of 48 606) and 92·03% (44 734 of 48 606), respectively (figure 1A). When broken down into cancer types by anatomical site of origin, the prevalence of MSS–TMB-H ranged from 0·10% to 13·17%, with the highest in anal carcinoma (13·17% [88 of 668]), followed by oesophageal squamous cell carcinoma (10·01% [81 of 809]), whereas the lowest was seen in gastrointestinal stromal tumours (0·10% [one of 1002]; figure 1B).

Gene mutations were most common in the dMMR/MSI-H subgroup, followed by the MSS–TMB-H and MSS–TMB-L subgroups (appendix p 3). Frameshift mutations were enriched in the dMMR/MSI-H subgroup, while nonsense mutations were enriched in the MSS–TMB-H subgroup (appendix p 4). The MSS–TMB-H subgroup carried the exclusively highest mutation rates in *POLE* (MSS–TMB-H *vs* dMMR/MSI-H *vs* MSS–TMB-L: 6·97% [111 of 1593] *vs* 0·66% [15 of 2270] *vs* 0·01% [four of 44 625]); *XPO1* (2·09% [32 of 1530] *vs* 0·09% [two of 2179] *vs* 0·04% [15 of 42 620]); *SMAD2* (4·77% [75 of 1571] *vs* 2·83% [64 of 2264] *vs* 1·38% [606 of 43 790]); *ERBB4* (1·14% [18 of 1582] *vs* 0·40% [nine of 2265] *vs* 0·16% [69 of 44 305]); *MAP2K1* (2·63% [42 of 1599] *vs* 1·45% [33 of 2272] *vs* 0·74% [331 of 44 709]); *MTOR* (2·01% [32 of 1592] *vs* 0·75% [17 of 2267] *vs* 0·54% [241 of 44 518]); *NFE2L2* (1·90% [27 of 1422] *vs* 0·24% [five of 2111] *vs* 0·54% [220 of 40 647]); and *TERT* (5·15% [42 of 815] *vs* 0·94% [nine of 953] *vs* 2·99% [574 of 19 177]), compared with the dMMR/MSI-H and MSS–TMB-L subgroups (q<0·0001, fold change >1·5; figure 2A); these eight genes were defined as gene panel A. With

the increase of the TMB cutoff, *POLE* mutations were the most prevalent (mutation rate 77·94% [106 of 136] for TMB ≥50 mutations per megabase and 43·82% [110 of 251] for TMB ≥20 mutations per megabase) in the MSS–TMB-H subgroup (appendix p 5). The frequencies of gene mutations in *PIK3R1, CDC73, JAK2, RB1, MAP2K4, CTNNA1, EZH2, KEAP1, RUNX1, PPP2R2A, PPP2R1A, STAG2, PTPN11, RAF1,* and *RASA1* (15 genes defined as gene panel B) were similar between the dMMR/MSI-H and MSS–TMB-H subgroups, but significantly lower in the MSS–TMB-L subgroup (q<0·0001, fold change >3; figure 2B; appendix pp 20–21). In *RNF43, ARID1A*, *MSH3*, *KMT2D*, and *ASXL1* (the top five genes in terms of statistical significance of between-subgroup differences), the MSS–TMB-H subgroup exhibited significantly lower mutation rates than the dMMR/MSI-H subgroup, but significantly higher mutation rates than the MSS–TMB-L subgroup (q<0·0001; figure 2C; appendix pp 20–21).

Copy number amplifications were more prevalent in both MSS subgroups than in dMMR/MSI-H tumours (appendix p 6); tumours with MSS–TMB-H carried a significantly higher rate of amplification in *CCND1* (MSS–TMB-H *vs* dMMR/MSI-H *vs* MSS–TMB-L: 3·74%

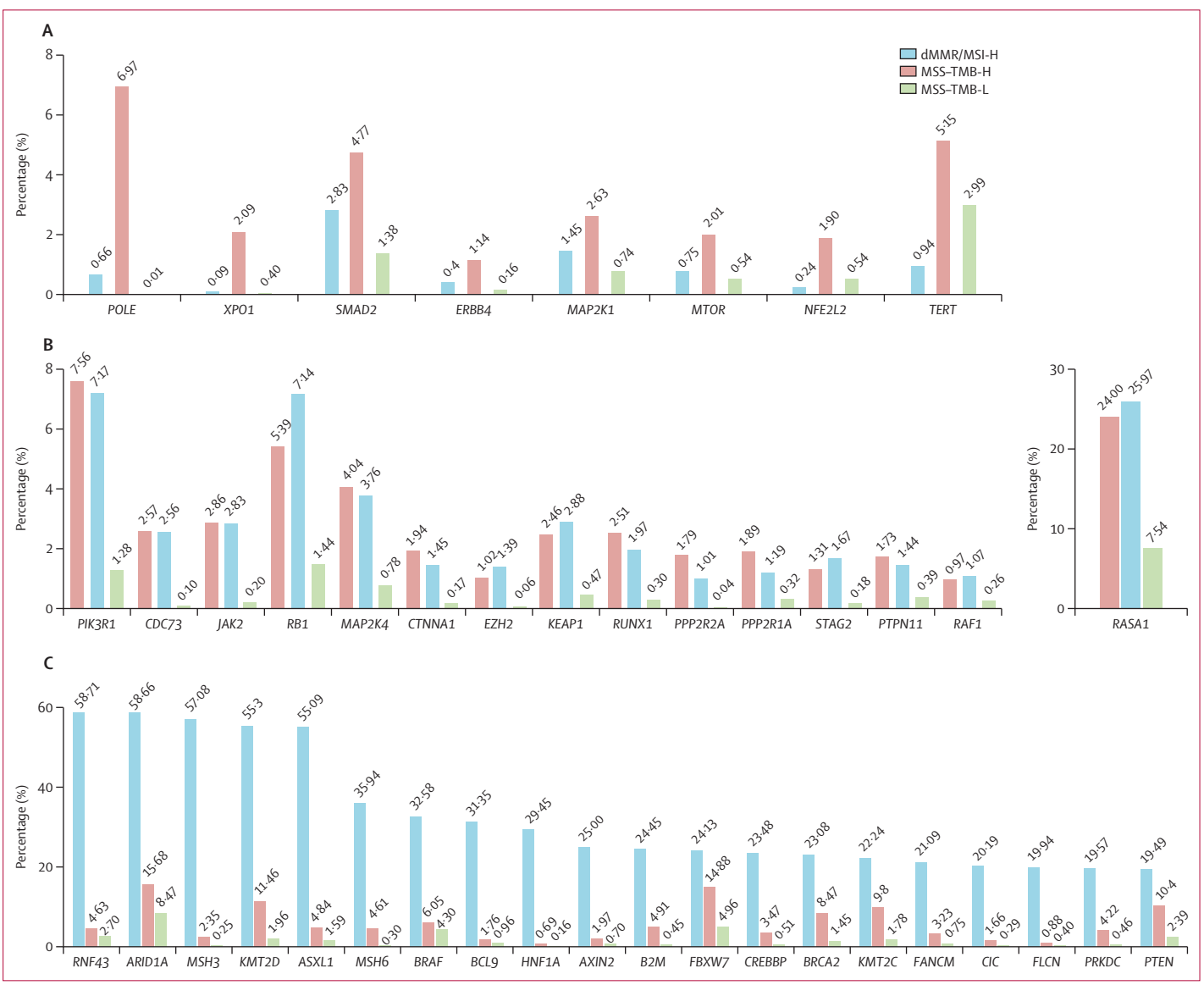

Figure 2: **Distinct genomic mutations among dMMR/MSI-H, MSS–TMB-H, and MSS–TMB-L subgroups in gastrointestinal cancers in the CARIS cohort**
(A) The landscape of gene mutations related to TMB-H, independent of dMMR/MSI-H (all q<0·0001, fold change >1·5, ranked by q value). (B) The landscape of genes with similar mutation rates between dMMR/MSI-H and MSS–TMB-H subgroups, but with significantly lower mutation rates in the MSS–TMB-L subgroup (all q<0·0001, fold change >3, ranked by q value). (C) The landscape of genes with significantly lower mutation rates in the MSS–TMB-H subgroup than in the dMMR/MSI-H subgroup, but significantly higher mutation rates than in the MSS–TMB-L subgroup (all q<0·0001, ranked by q value). dMMR=mismatch-repair deficiency. MSI-H=microsatellite instability-high. MSS=microsatellite stable. TMB-H/L=tumour mutational burden-high/low.

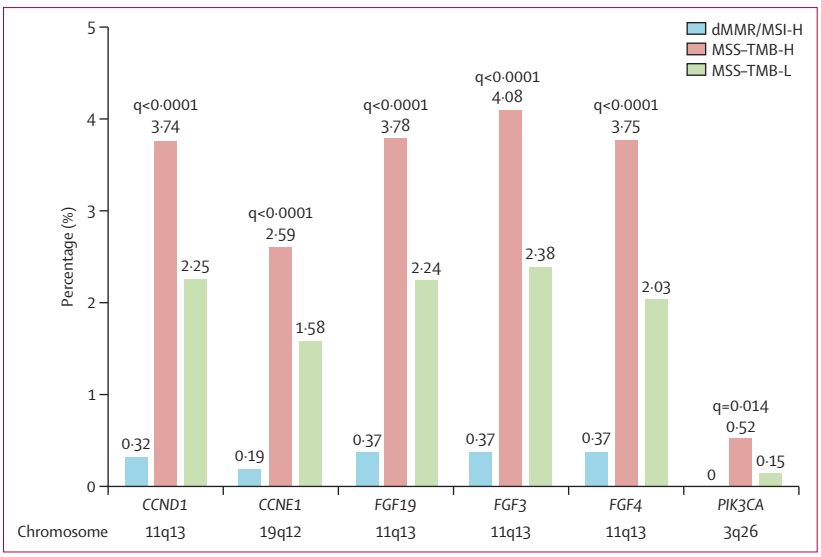

***Figure 3*: Distinct features of copy number amplifications among dMMR/MSI-H, MSS–TMB-H, and MSS–TMB-L subgroups in gastrointestinal cancers in the CARIS cohort (ranked by q value, q<0·05)**
dMMR=mismatch-repair deficiency. MSI-H=microsatellite instability-high. MSS=microsatellite stable. TMB-H/L=tumour mutational burden-high/low.

[58 of 1552] *vs* 0·32% [seven of 2192] *vs* 2·25% [972 of 43 280]; q<0·0001); *CCNE1* (2·59% [40 of 1542] *vs* 0·19% [four of 2155] *vs* 1·58% [675 of 42 842]; q<0·0001); *FGF19* (3·78% [58 of 1534] *vs* 0·37% [eight of 2167] *vs* 2·24% [955 of 42 688]; q<0·0001); *FGF3* (4·08% [63 of 1518] *vs* 0·37% [eight of 2135] *vs* 2·38% [995 of 41739]; q<0·0001); *FGF4* (3·75% [58 of 1548] *vs* 0·37% [eight of 2184] *vs* 2·03% [873 of 42 942]; q<0·0001); and *PIK3CA* (0·52% [eight of 1539] *vs* 0% [0 of 2175] *vs* 0·15% [64 of 42 981]; q=0·014), compared with the dMMR/MSI-H and MSS–TMB-L subgroups (figure 3). Most of them (67% [four of six]) are located on chromosome 11q13.

Gene fusions in *NTRK1*, *ALK*, *RET*, and *NTRK3* were rare in both the MSS–TMB-H and MSS–TMB-L subgroups, and were highest in the dMMR/MSI-H subgroup. However, *FGFR2* fusions were more prevalent in the MSS–TMB-L subgroup than in the dMMR/MSI-H and MSS–TMB-H subgroups (figure 4A). HER2 positivity evaluated by immunohistochemistry and CISH was highest in the MSS–TMB-H subgroup, followed by the MSS–TMB-L and dMMR/MSI-H subgroups; this finding was confirmed by copy number analysis (figure 4B). PD-L1 expression was highest in the dMMR/MSI-H subgroup, followed by the MSS–TMB-H and MSS–TMB-L subgroups (figure 4C).

We further explored the impact of genes from panels A and B on the tumour immune microenvironment based on the TCGA database. *NFE2L2* and *TERT* mutations significantly increased fraction of genome alterations (p<0·0001) and intratumour heterogeneity (p=0·0023), respectively, in the MSS subgroup (appendix p 7), which might contribute to the resistance to immune checkpoint inhibitors.[15] The immune cell infiltration analyses suggested that *MTOR*, *KEAP1*, *NFE2L2*, *RASA1*, *RB1*, and *SMAD2* were predicted to be negatively associated with antitumour immune response (eg, the decrease of natural killer [NK] T cells [*NFE2L2*, p<0·0001], activated NK cells [*MTOR*, p=0·0064; *SMAD2*, p=0·0072], $CD8^+$ T cells [*RASA1*, p=0·019; *RB1*, p=0·0015; *SMAD2*, p=0·0027], and T-helper-1 [Th1] cells [*NFE2L2*, p=0·025; *RB1*, p=0·018]; and the elevation of M2-like macrophages [*KEAP1*, p=0·0012] and neutrophils [*MTOR*, *p*=0·022]; appendix p 7). In addition, *RB1* and *NFE2L2* mutations are associated with the downregulation of immunoreactive signature scores (eg, *RB1*: cytolytic activity, p=0·0056, effective T-cell score, p=0·016, T-cell-inflamed gene expression profile, p=0·0069, lymphocyte infiltration signature score, p=0·0017; *NFE2L2*: lymphocyte infiltration signature score, p=0·018; appendix p 7). These results indicated that *MTOR*, *TERT*, *KEAP1*, *NFE2L2*, *RB1*, *RASA1* and *SMAD2* mutations might impair antitumour immune response in MSS gastrointestinal tumours, despite the association with TMB-H. Therefore, we evaluated 16 other genes associated with an active tumour immune microenvironment (*CDC73*, *CTNNA1*, *ERBB4*, *EZH2*, *JAK2*, *MAP2K1*, *MAP2K4*, *PIK3R1*, *POLE*, *PPP2R1A*, *PPP2R2A*, *PTPN11*, *RAF1*, *RUNX1*, *STAG2*, and *XPO1*) to construct an mTMB gene signature for efficacy of immune checkpoint inhibition in MSS gastrointestinal tumours.

MSS gastrointestinal tumours with a mutation in any gene in the mTMB signature displayed significantly higher mutation counts (p<0·0001), indel (p<0·0001) and single nucleotide variant (p<0·0001) neoantigens, and B-cell receptor richness (p=0·024) compared with mTMB wildtype tumours in the TCGA cohort (figure 5A–C). Gene mutations in any gene in the mTMB signature were also associated with high interferon gamma (IFNγ) signature scores (p=0·0073) and high infiltration of activated NK cells (p=0·013), M1-like macrophages (p=0·0002), and Th1 cells (p=0·013), but low infiltration of M2-like macrophages (p=0·014; figure 5D–E, appendix p 9). Of note, TMB in MSS tumours with a mutation in *MTOR*, *TERT*, *KEAP1*, *NFE2L2*, *RB1*, *RASA1*, or *SMAD2* was significantly lower than in tumours with any mutation in the mTMB signature but was higher than in those with wildtype mTMB (appendix p 8). Basic characteristics of patients with gastrointestinal cancers who received immune checkpoint inhibitor treatment in the CARIS, MSKCC, and PUCH cohorts, as well as those who did not in the TGCA cohort, are shown in the appendix (pp 14, 15); ethnicity data were not collected for our study. Among immune checkpoint inhibitor-treated patients in the CARIS cohort, after a median follow-up of 22·10 months (IQR 7·20–31·30), patients with any mutation in the mTMB signature (n=42) exhibited significantly longer overall survival than patients with mTMB wildtype tumours (n=53; median 18·77 months [95% CI

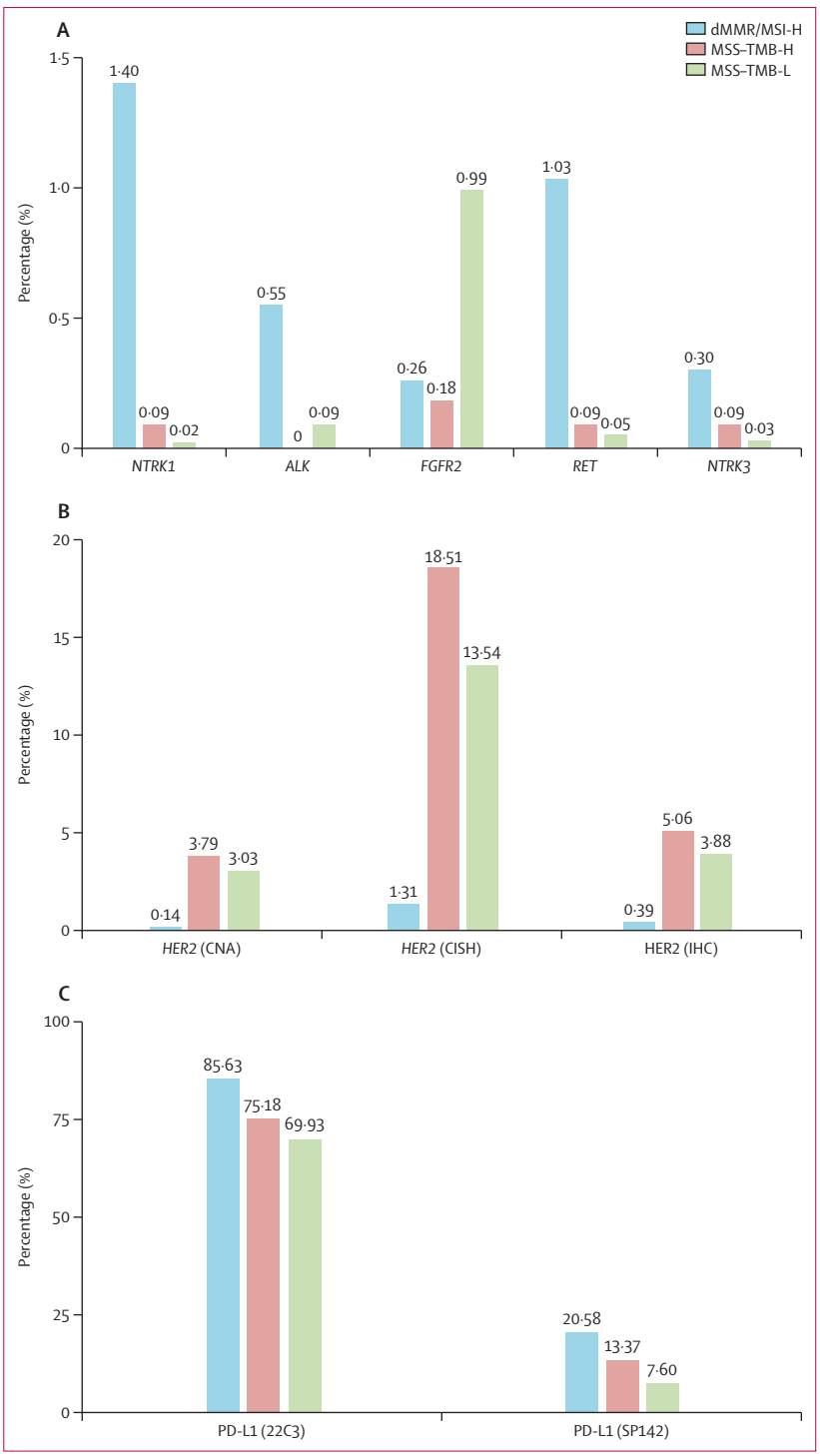

Figure 4: **Differences in gene fusions, HER2 status, and PD-L1 expression among dMMR/MSI-H, MSS–TMB-H, and MSS–TMB-L subgroups in gastrointestinal cancers in the CARIS cohort**

(A) Comparison of actionable gene fusions among MSS–TMB-H, dMMR/MSI-H, and MSS–TMB-L subgroups (all p<0·0001). (B) Comparison of HER2 positivity evaluated by CISH, IHC, and next-generation sequencing (copy number analysis) among MSS–TMB-H, dMMR/MSI-H, and MSS–TMB-L subgroups (all p<0·0001). (C) Comparison of PD-L1 expression among MSS–TMB-H, dMMR/MSI-H, and MSS–TMB-L subgroups (all p<0·0001). For PD-L1 expression, the Dako Link 48 platform for the Dako 22C3 pharmDx kit (SK006; Agilent, Santa Clara, CA, USA) and the Ventana BenchMark ULTRA platform for the SP142 assay kit (740-4859; Ventana, Oro Valley, AZ, USA) were used for gastro-oesophageal cancer and other gastrointestinal cancers, respectively. CISH=chromogenic in situ hybridisation. CNA=copy number analysis. dMMR=mismatch-repair deficiency. IHC=immunohistochemical staining. MSI-H=microsatellite instability-high. MSS=microsatellite stable. TMB-H/L=tumour mutational burden-high/low.

17·30–20·23] *vs* 7·03 months [5·73–8·34]; hazard ratio [HR] 0·55 [95% CI 0·31–0·99], p=0·044; figure 5F). This association remained significant in the sensitivity analysis including only patients ranked in the top 95% of longest follow-up time in the cohort (median follow-up 22·10 months [IQR 7·23–31·30]; median overall survival 18·77 months [95% CI 17·32–20·22] *vs* 7·03 months [5·58–8·48], HR 0·49 [95% CI 0·27–0·92], p=0·024; appendix p 10). A similar result (HR 0·47 [95% CI 0·30–0·74], p=0·0018) was also observed in the MSKCC cohort (appendix p 11), but MSI-H status was not available for this cohort, which limited the analysis. No significant association between any mutation in the mTMB signature and overall survival was observed in the PUCH cohort (HR 0·54 [95% CI 0·19–1·54], p=0·32), probably due to the small sample size (appendix p 11). Of interest, at a median follow-up of 24·80 months (IQR 14·12–43·23), there was no significant association between gene mutations in the mTMB signature and overall survival benefit in patients in the TCGA cohort who never received immune checkpoint inhibitor treatment (median overall survival 54·07 months [95% CI 41·24–55·96] *vs* 48·60 months [34·51–73·62], HR 0·94 [95% CI 0·74–1·20], p=0·61; appendix p 12). The potential mechanism of the mTMB signature leading to the increased efficacy of immune checkpoint inhibitor treatment is shown in the appendix (p 13).

Discussion

To the best of our knowledge, this is the largest study to explore the molecular features of MSS gastrointestinal tumours with TMB-H. In our study, the prevalence of TMB-H in MSS gastrointestinal cancers was 3·29%, lower than in Goodman and colleagues' study (43 different histologies), in which it was 5·36% (7972 of 148 803 tumour samples).[16] This discrepancy is probably due to the heterogeneity of population, the composition of cancer types, technological issues, and the different TMB algorithm. However, up to now, no sufficient evidence has shown that MSS gastrointestinal tumours with a TMB of ten or more mutations per megabase can benefit from immune checkpoint inhibitor treatment. Efforts to determine TMB accurately and adopt an optimal TMB threshold in a given cancer type are ongoing.

The correlation between TMB and the formation of immunogenic neoantigens might partly depend on

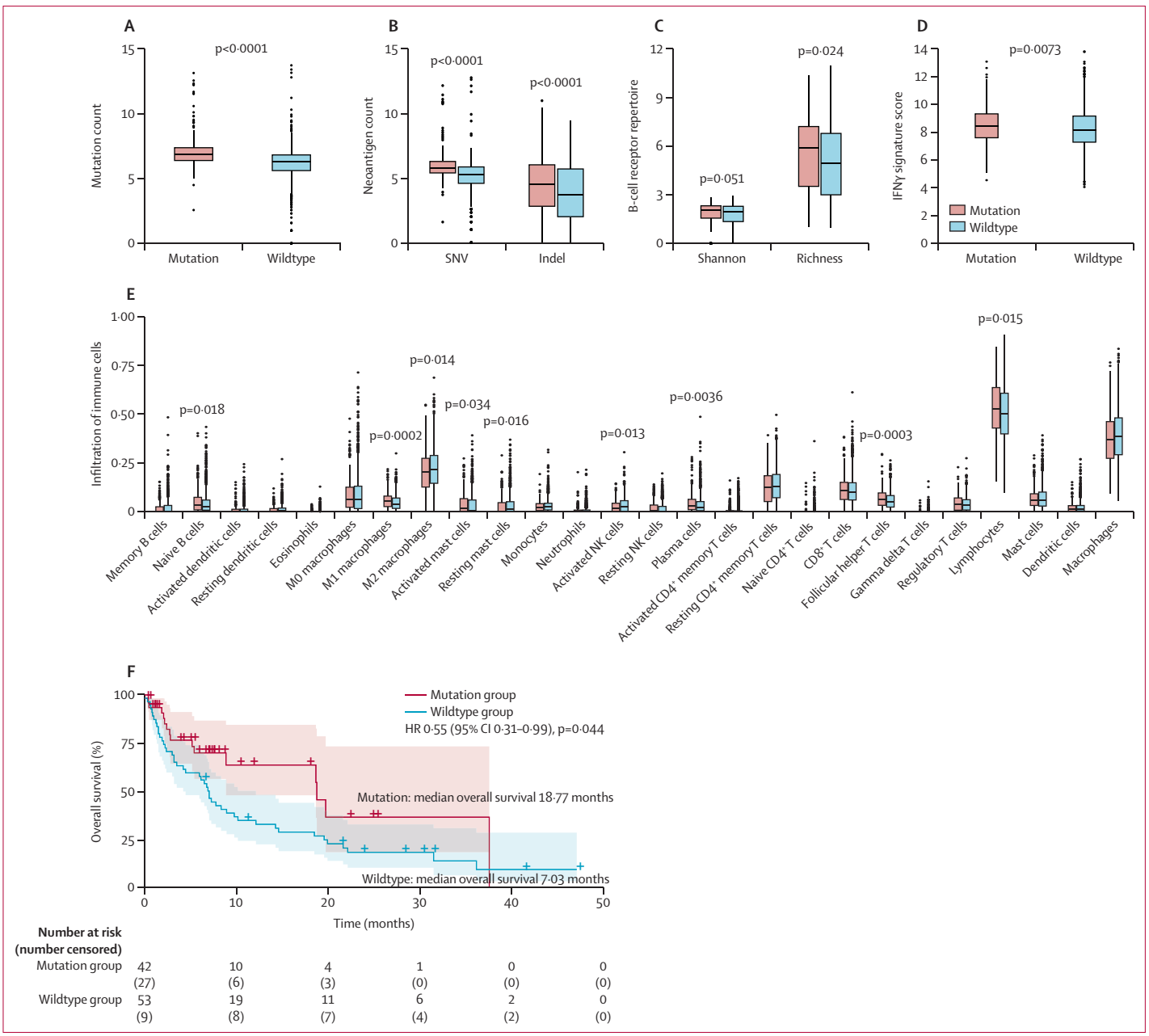

Figure 5: **Impact of gene mutations in the mTMB signature on tumour immune environment in the TCGA cohort and immune checkpoint inhibitor efficacy in the CARIS cohort in gastrointestinal cancers**

(A) Difference in mutation counts (log2 transformed) between gastrointestinal tumours in the CARIS cohort with and without gene mutations in the mTMB signature. (B) Difference in neoantigen counts (log2 transformed) between gastrointestinal tumours in the CARIS cohort with and without gene mutations in the mTMB signature. (C) Difference in B-cell receptor repertoires (Z scores, log2 transformed) between gastrointestinal tumours in the CARIS cohort with and without gene mutations in the mTMB signature. (D) Association of mTMB signature with immune signature in gastrointestinal cancers in the CARIS cohort. (E) Impact of gene mutations in the mTMB signature on the infiltration of immune cells (the proportions of immune cells within the leukocyte compartment) in gastrointestinal cancers in patients in the TCGA cohort, estimated using CIBERSORT. (F) Impact of gene mutations in the mTMB signature on overall survival in patients with MSS gastrointestinal cancers who received immune checkpoint inhibitors in the CARIS cohort. The shaded areas represent 95% CIs. HR=hazard ratio. IFNγ=interferon gamma. MSS=microsatellite stable. mTMB=modified tumour mutational burden. NK=natural killer. TCGA=The Cancer Genome Atlas. SNV=single nucleotide variant.

the mutational signatures, causing immune checkpoint inhibitor response or resistance. Our study suggests that it might not be the quantity of mutations but rather the quality of mutations generating immunogenic neoantigens. Not all the genes associated with TMB-H could improve antitumour immune response. We found that gene mutations in *SMAD2, MTOR, KEAP1, NFE2L2, RB1, TERT,* and *RASA1* were associated with negative predictors of immune checkpoint inhibitor efficacy (eg, tumour heterogeneity and fraction of

genome alterations), lower infiltration of immunoreactive cells, or the inactivation of immune-related pathways, despite high TMB. *SMAD2* is related to the restrained tumour-killing effect and the limited infiltration of immune effector cells.[17] The loss of RB1 can reduce immune cell mobilisation and antigen presentation.[18] Mutations in the *KEAP1–NFE2L2* pathway might protect tumour cells from oxidative stress, promoting tumour growth and aggressiveness.[19] Although *KEAP1–NFE2L2* mutations are associated with TMB-H, the deficient infiltration of $CD4^+$ T cells, NKT cells, and regulatory T cells was observed in *KEAP1–NFE2L2*-mutant non-small-cell lung cancers.[20] *TERT* mutations were reported to be a negative predictor of immune checkpoint inhibitor efficacy in metastatic renal cell carcinoma.[21] The association of mutations in *RASA1* and *MTOR* with TMB and immune checkpoint inhibitor efficacy are not well studied so far. Based on our study, *RASA1* mutations were related to lower infiltration of $CD8^+$ T cells. *MTOR* mutations might reduce the infiltration of activated NK cells. Meanwhile, genes in the mTMB signature were predicted to be associated with enhanced anti-tumour immune response, indicated by the increased infiltration of active immune cells (eg, M1-like macrophages, Th1 cells, NKT cells, and so on). We observed that patients with any mutation in the mTMB signature have a significant overall survival benefit when treated with immune checkpoint inhibitors, but this was not the case in patients receiving chemotherapy or surgery in the TGCA cohort. In particular, mutations in *POLE*, which provides an important proofreading function during DNA replication, lead to a distinct hypermutated but MSS phenotype with improved immune checkpoint inhibitor efficacy. In non-small-cell lung cancers, mutations in *XPO1* and *ERBB4* are positively associated with TMB-H and high PD-L1 expression, which resulted in higher response rates to immune checkpoint inhibitors.[22,23] *MAP2K1, MAP2K4, RAF1,* and *PTPN11* mutations can lead to the activation of the MAPK pathway, which we have previously shown to increase benefit from immune checkpoint inhibitor treatment in patients with gastro-oesophageal adenocarcinomas.[24] The inactivation of EZH2 can sensitise tumours to immune checkpoint inhibitors by improving effector functions of $CD8^+$ T cells, promoting IFNγ production and cytotoxicity.[25] *JAK2* loss-of-function mutations, such as nonsense mutations, were considered to be a predictive marker for hyperprogression; however, emerging evidence has shown that *JAK2* missense mutations, especially V617F, could confer sensitivity to immune checkpoint inhibitor treatment.[26] In our study, we did not include *JAK2* nonsense mutations in the mTMB signature. The association between various mutation sites of *JAK2* and immune checkpoint inhibitor efficacy should be explored further. The inactivation of PP2A (encoded by *PPP2R1A* and *PPP2R2A*) could convert so-called cold MSS tumours (ie, those with a lack of T cells infiltrating the tumour) into MSI tumours via triggering neoantigen production, cytotoxic T-cell infiltration, and immune checkpoint inhibitor sensitisation.[27] STAG2 (also known as cohesin subunit SA-2) is associated with genomic stability. STAG2 deficiency might induce interferon response and PD-L1 expression, leading to the increased response to immune checkpoint inhibitors in melanoma.[28] Mutations in *PIK3R1* might lead to the activation of the PI3K/AKT pathway, which has been reported as a primary resistance mechanism to immune checkpoint inhibitors in MSI-H gastrointestinal cancers.[29] By contrast, in MSS colorectal cancer, *PIK3CA* mutations are associated with increased cytotoxic T-cell infiltration, higher PD-L1 expression, and greater clinical benefit from immune checkpoint inhibitors,[30] highlighting that the regulation of tumour immunity by the PI3K/AKT pathway is context-dependent. No studies on the association between *CTNNA1, CDC73, RUNX1,* and immune checkpoint inhibitor efficacy have been reported. Based on their biology, it has been suggested that *CDC73* and *RUNX1* are involved in RNA polymerase II transcription;[31,32] thus, we speculated that mutations in *CDC73* and *RUNX1* might increase transcriptional stress and genomic instability. However, not all the gene mutations in the mTMB signature are equally able to induce efficient neoantigens presented by an MHC. More in-depth research efforts about the optimisation of predictive models for immune checkpoint inhibitor treatment are warranted.

Copy number alteration burden is negatively associated with immune checkpoint inhibitor efficacy. Patients with high TMB and low copy number alteration cancer can be an optimal subgroup for immune checkpoint inhibitor therapy in gastrointestinal cancers.[33] Immune checkpoint inhibitor treatment might have inferior efficacy in oesophageal squamous cell cancers with chromosome 11q13 amplification compared with those without chromosome 11q13 amplification.[34] Mechanically, chromosome 11q13 amplifications might impair the antitumour activity of immune checkpoint inhibitors, indicated by the decrease in $CD8^+$ T-cell, NKT-cell, and B-cell infiltration.[35] In our study, we found that copy number amplification was more prevalent in MSS tumours than in dMMR/MSI-H tumours, especially in chromosome 11q13 (*CCND1, FGF3, FGF4,* and *FGF19*), strengthening the rationale for combination therapies with agents targeting cyclin D1 (CCND1) and FGFs with immune checkpoint inhibitors. In addition, our data also showed that MSS gastrointestinal tumours with TMB-H have a higher frequency of *HER2* amplification compared with dMMR/MSI-H and MSS–TMB-L gastrointestinal tumours, which might provide one theoretical explanation for the enhanced efficacy of combining immune checkpoint inhibitors with anti-HER2 treatment.

Limitations of this work need to be mentioned, including its retrospective nature and the heterogeneity

of cancer types between different cohorts. Due to the population of the MSS–TMB-H subgroup, we had to analyse all the cancer types together. It would be worthwhile to explore the genomic signatures related to TMB-H and the association with immune checkpoint inhibitor efficacy in each cancer type separately. Second, due to the small sample size and the absence of some types of clinical information (eg, T-cell infiltration density, C-reactive protein concentrations, and antibiotic treatment) in the cohorts of patients treated with immune checkpoint inhibitors in our study, which might influence immune checkpoint inhibitor efficacy, prospective randomised studies with a larger sample size of gastrointestinal cancers are warranted to further confirm the predictive value of the mTMB signature for immune checkpoint inhibitor efficacy and its interaction with the tumour immune microenvironment by integrated analyses of multiple omics.

In conclusion, our data suggest that not all the mutations related to TMB-H can improve antitumour immune response, and specific gene mutations, such as *SMAD2, MTOR, KEAP1, NFE2L2, RB1, TERT,* and *RASA1* should also be taken into consideration. The combination of TMB and gene mutations positively regulating antitumour immunity (such as mTMB) might be a promising tool for patient selection for immune checkpoint inhibitor treatment. Our data also provide novel insights for the combination of drugs targeting cyclin D1 or FGFs and immune checkpoint inhibitor treatment.

Contributors

JW, JX, WMK, and H-JL initiated and conceived the study. JW, JX, YB, AF, AFS, AG, BAW, JLM, EL, MK, DPSS, MJH, FB, HA, NK, SS, WZ, MO, DS, TL, LS, WMK, and H-JL assisted in sample collection, acquiring and managing patients, and providing facilities. JW, JX, AF, YB, WZ, and H-JL developed the analysis plan. JW wrote the first draft of the manuscript. JX, AF, YB, MO, DS, and WMK accessed and verified the raw data of the CARIS cohort used in this study and processed data with JW. H-JL supervised the study. All authors contributed to data interpretation and writing and approved the final manuscript for publication. All authors had full access to all the analysed data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

H-JL reports receiving honoraria from serving as a consultant or from advisory board membership for Merck Serono, Bayer, and Genentech. JX, AF, YB, MO, DS, and WMK are employees of Caris Life Sciences. AFS reports funding for research, travel, and speakers bureau participation from Caris Life Sciences. BAW reports receiving honoraria from Bayer, Sirtex, Lilly, Taiho, and HalioDx. All other authors declare no competing interests.

Data sharing

Data (including the summary of clinical and genomic data) will be made available upon reasonable request with the permission of Caris Life Sciences. Data can be requested by contacting the corresponding author. Individual participant data of the CARIS cohort from this study are not available for sharing.

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