Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Rousseau B, Foote MB, Maron SB, et al. The spectrum of benefit from checkpoint blockade in hypermutated tumors. N Engl J Med 2021;384:1168-70. DOI: 10.1056/NEJMc2031965

SUPPLEMENTARY APPENDIX

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Supplemental Methods:

Patient Selection and Data Sources

Clinical and genetic data from patients followed at Memorial Sloan Kettering Cancer Center (MSKCC) with colorectal cancer (CRC) who underwent testing with the next generation sequencing targeted "MSKCC IMPACT" panel of somatic mutations[1] (341, 410, 468 and 505 gene coverage analyzed over time in successive panels) was obtained from an internal database (N=4283, Supplemental Table S1, Supplementary Figure S1). All included patients consented to the MSKCC protocol IRB 12-245.

Patients who consented to IRB 12-245 and received PD-1/PD-L1 (pembrolizumab,nivolumab, atezolizumab, durvalumab, avelumab) or CTLA-4 (tremelimumab, ipilimumab) targeted therapy prior to 11/27/2019 with documented malignancy of the colon or rectum were identified from the Memorial Sloan Kettering institutional database (Supplementary Figure S1, Supplementary Table S2). Clinical data, including demographics, cancer characteristics (histology, primary tumor location, staging), and prior treatment details were extracted from the electronic medical record (EMR) and verified by licensed physicians (S.M, B.D: Supplemental Table S2). Guidelines for abstraction were shared in a joint file among team members, with specific instructions to accurately code each data point. Patient clinical characteristics are displayed in Table S2. The study was approved by the MSKCC institutional review board (IRB 2020-013).

A separate, publicly available validation cohort of patients with metastatic cancer of different primary tumors treated with immune checkpoint inhibitors (ICIs; n=1661) from 2015 to 2019 was also assessed. Data from this cohort has been previously reported[2]. This study was IRB-approved at MSKCC. Given the large array of tumor types assessed in this cohort, similar tumor types were consolidated into larger categories according to tumor primary site and histology. All patients in this cohort received at least one dose of ICI, including anti PD-1/PD-L1, anti CTLA4 or a combination of two ICIs (Supplementary Table S5). Clinical information was compiled as described previously[2]. This dataset was supplemented with

additional information acquired from an internal MSKCC institutional database, including the mismatch-repair-deficiency (MMRd), *POLD1* and *POLE* pathogenic mutations affecting the proofreading domain (*POLD1* or *POLE* proofreading defect is designated as *pol-d*) for each patient (as described below). Clinical and genetic data are available at reasonable request to the corresponding author.

Genetic Mutational Analysis and TMB Assessment

Study patients in both the colorectal cancer cohort and the second multi-tumor type validation cohort[2] all underwent genetic sequencing with the MSKCC IMPACT panel[1]. Tumor mutational burden was calculated for each patient in both cohorts by dividing the total number of non-synonymous somatic mutations (single nucleotide variants and insertions/deletions) by the total megabase coding region coverage. Of note, the MSK IMPACT TMB score has been shown to highly correlate with other methods of genetic sequencing assessment, including Whole Exome Sequencing and the FoundationOne Medicine CDx assay used as the companion diagnostic tool in the KEYNOTE 158 trial[2-3]. Patients with incomplete TMB calculations in the parent cohort of patients with advanced colorectal cancer (N=26 out of 4283) were not included in the final analyzed experimental population. The mutational status of several genes, including *KRAS*, *NRAS*, and *BRAF*, were also assessed for patients with colorectal cancers. Only mutations with high functional impact according to the MSK OncoKB Precision Oncology database were considered to be pathologic[4]. Polymerase ε(*POLE*) and polymerase δ1 (*POLD1*) proofreading defect status (together defined as *pol-deficient* or *pol-d*) in each patient was defined by two independent physicians (B.R and M.F). Patients were designated as exhibiting tumor *pol-d* status if their tumor contained a polymerase mutation associated with pathogenesis by Campbell et al[5] or the OncoKB[4] validated genetic dataset. In addition, patient tumors were defined as *pol-d* if the respective polymerase mutation was a non-synonymous missense mutation of unknown significance within the exonucleasic domain compatible as described by Rousseau et al[6]. POLD1 sequencing was added recently to the IMPACT panel, therefore a subgroup of patients had a missing POLD1 status (N=259/1661). POLD1 pathogenic mutations were found only in MMRd patients, while

POLE pathogenic mutations were observed only in MMRp patients. Therefore, for the MMRp patients with missing POLD1 status, the *pol-d* status was determined solely according to the POLE mutational profile. MMRd and MMRp *pol-d* patients were combined in the analysis as "MMRd or *pol-d*" representing patients with a DNA repair defect.

Determination of Mismatch-Repair-Deficiency Status

For patients in both the advanced CRC patient cohort and the second multi-tumor type validation cohort, MMRd status, also noted as "microsatellite instability" (MSI), was confirmed using immunohistochemistry (IHC) and/or by analyzing a clinically-validated MSI score calculated from IMPACT targeted panel sequencing using the MSIsensor program[7]. An MSI impact score ≥10 was sufficient to classify the patient as MSI-high/MMRd as previously described[7]. Patients with an MSI impact score <10 were classified as mismatch-repair-proficient (MMRp). Patients with an indeterminate MMR status according to IHC or IMPACT were categorized according to the above thresholds defined by the MSI sensor score. Patients with indeterminate IHC, indeterminate targeted panel sequencing MMR-status, and a missing MSI score were characterized as indeterminate and were not included in the primary study cohorts (N=26 out of 4283).

Based on this determination, patients in the advanced CRC cohort were identified in one of four categories based on a combination of DNA-repair defect status and TMB: (1) MMRp and TMB<10 , (2) MMRp and TMB≥10, (3) MMRd, and (4) *pol-d* (Table S2). Patients with metastatic colorectal cancer from the larger MSKCC IMPACT colorectal cohort (described in Table S1) who were not treated with ICI were also delineated into the same categories (Table S7). In the second multi-tumor type validation cohort, patients were similarly parsed into one of three categories based on the same information: (1) MMRp and TMB<10, (2) MMRp and TMB≥10, and (3) MMRd or *pol-d* (Table S5). These patient subgroups for each study cohort were discrete and no patient belonged to more than one subgroup.

Study Outcomes

Clinical outcomes for patients in the advanced CRC patient cohort were assessed using physician notes from the EMR and radiologic scan reports. Radiologic scan reports from computed tomography (CT), magnetic-resonance-imaging (MRI), or Positron Emission Tomography – Computed Tomography (PET-CT) imaging were assessed throughout each patient's course of therapy. For the advanced CRC patient cohort exposed to ICI, progression-free-survival (PFS) was calculated for each patient using Kaplan-Meier analysis starting at the documented EMR date of first dose of the ICI with the end date as either documented date of patient death or the patient imaging scan demonstrating disease progression as per the Response Evaluation Criteria in Solid Tumours (RECIST)[8]. Overall Survival (OS) for patients in the different cohorts was calculated in similar fashion using the documented EMR start date for the respective patient ICI treatment and date of documented patient death in the medical record, death note, and/or telephone encounters with family regarding outside hospitalization. In the absence of the relevant event for survival outcome, patients were censored at the date of last follow up. One-year and 2-year overall survival and progression-free survival rates were calculated for all patients with advanced colorectal cancer using Kaplan-Meier analysis (depicted in Supplemental Table S3 and S6, respectively).

In a sensitivity analysis, median survival from date of metastatic diagnosis (based on EMR radiographic documentation of metastases or confirmed metastasis biopsy) to either documented date of patient death or last follow-up (censored at that moment) was performed using Kaplan-Meier analysis for patients with advanced colorectal cancer who were or were not treated with ICI (Supplementary Table S7). In addition, the median time from metastatic diagnosis to date of first ICI treatment was also similarly calculated for patients with advanced CRC who were treated with ICI (Supplementary Table S7). Patients without a precise date of metastatic diagnosis in the EMR (10 out of 137 patients with advanced CRC treated with ICI) were excluded from the sensitivity analysis.

Statistical Analysis

In the advanced colorectal cancer cohort, patients were stratified first by TMB status greater than or less than 10, and subsequently hazard ratios (HRs) were established for the association between the incidence of progression or death (PFS: Supplementary Table S6) and death (OS: Supplementary Table S3) between these two TMB groups using univariate Cox proportional hazards regression modeling. All Cox models utilized the onset date of first ICI treatment and the primary endpoint of date of progression or death (Supplementary Table S6) or death (Supplementary Table S3). Patients from this cohort were then stratified into four factor variable levels based on the four TMB/DNA-repair defect subgroups defined above ("Determination of Mismatch-Repair-Deficiency Status") and HRs assessing the association between presence of each TMB/DNA-repair defect subgroup status and progression or death (PFS: Supplemental Table S6) or death (OS: Supplemental Table S3) were also established using Cox Proportional Hazards Regression. The patient MMRp TMB<10 subgroup was used as a reference cohort for the calculation of every HR. Additional clinical covariates were also assessed using this same univariate analysis strategy (Supplemental Table S3 and S6). Notably, for the univariate immune checkpoint inhibitor treatment analysis, only patients with a single versus combination checkpoint inhibitor therapy were included; patients treated with a checkpoint inhibitor and non-checkpoint inhibitor therapy (including a tyrosine kinase inhibitor or chemotherapy, N=42) were not included in this specific univariate analysis but were included in the other univariate and multivariate analyses. Clinical covariates with a univariate HR associated with progression or death (based on 95% CI not overlapping 1.0) were added to multivariate models for the respective endpoint (PFS Supplementary Table S6, OS Supplementary Table S3) and a multivariate Cox Proportional Hazards regression was performed.

The proportional hazards assumption was verified for every Cox regression model using computerized modeling to assess a global value that represented culminated interactions between predictions and log(time), as well as visual Schoenfeld residual plots for each regression. The proportional hazards

assumption was confirmed in univariate Hazard Ratio calculations from the expanded cohort of patients with multiple malignancy types (Figure 1C, Supplementary Table S4), but was not maintained in the analysis depicted in Figure 1A where survival curves crossed in the advanced colorectal cancer cohort. A sensitivity analysis that stratified survival based on set time intervals and compared restricted mean survival times (RMST) between TMB subgroups (using data represented in Figure 1A) and DNA-Repair Status Subgroups (using data represented in Figure 1B) was performed (Table S9). Importantly, use of restricted time intervals did not highlight any significant difference at time intervals of 6, 12, 18, 24, and 30 months between patients with MMRp TMB<10 tumors and MMRp TMB≥10 tumors (from Figure 1B). The other associations seen in Figure 1A and 1B were maintained in this RMST sensitivity analysis.

For all analyses, censoring occurred at the time of loss of patient follow up or by 12/2/2019 (data freeze date) if the patient continued in the study without documentation of progression or death. Family-wise error rates and multiplicity were not adjusted in the analyses. Median follow-up times from start of ICI were calculated for all study cohorts and patient subgroups using Kaplan-Meier analysis (Supplementary Table S8).

A similar analysis was performed for patients in the second multi-tumor validation cohort. Patients in this cohort were classified by primary tumor type and then were similarly stratified initially by TMB status (greater than or equal to 10). A second analysis was performed in this cohort where patients were separated by primary tumor type and then stratified into one of three factor level subgroups (TMB<10, TMB≥10, DNA-repair defect (MMRd or *pol-d)*, Table S5), as described above ("Determination of Mismatch-Repair-Deficiency Status"). Cox Proportional Hazards Regression was performed for each analysis as above to establish HRs for an association between TMB status and death, and separately subgroup status and death (Figure 1C, Figure S3). In addition, an "Other Tumors" category was included that aggregated patients with tumor types where a HR for death was unable to be determined given respective insufficient numbers of TMB≥10 tumors. This category compiled patients with breast, kidney, neuroendocrine, uveal melanoma

and mucosal melanoma cancers to increase statistical power. The MMRp TMB<10 patient subgroup was used as the reference in each analysis.

Tumor types where the respective MMRp TMB≥10 patient subgroup is positively associated with overall survival (based on 95% CI not overlapping 1.0) were compiled into an aggregated subgroup (including patients with head and neck, melanoma and NSCLC tumors and labeled "Combined TMB Sensitive Tumors"; Supplementary Table S4 and Figure 1C). Cox Proportional Hazards regression modeling was performed in this aggregated cohort for the similar stratification by TMB and DNA-repair subgroups (Figure 1C, Supplementary Figure S3, Supplementary Table S4) to provide HR and 95% CI for the association between TMB or DNA-repair subgroup status and death, respectively. Tumor types where the respective MMRp TMB≥10 patient subgroup is not positively associated with overall survival (based on 95% CI not overlapping 1.0) were aggregated into a separate cohort and labeled "Combined TMB Insensitive Tumors" (Figure 1C, Supplementary Figure S3, Supplementary Table S4). Specific HRs for the association between each TMB/DNA-repair subgroup and death are shown per tumor type in Supplementary Table S4. Notably, in Table S4, HRs from patient subgroups with less than 3 patients were not displayed based on infinite confidence intervals and lack of observed events (labeled as "NE").

Supplementary References

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2. Samstein RM, Lee C-H, Shoushtari AN, et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat Genet 2019;51(2):202–6.

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6. ESMO. Nivolumab Shows Promising Activity in Advanced Tumours with Rare Mutations [Internet]. [cited 2020 Oct 14];Available from: https://www.esmo.org/oncology-news/nivolumab-shows-promising-activity-in-advanced-tumours-with-rare-mutations

7. Middha S, Zhang L, Nafa K, et al. Reliable Pan-Cancer Microsatellite Instability Assessment by Using Targeted Next-Generation Sequencing Data. JCO Precis Oncol [Internet] 2017 [cited 2020 Oct 16];1. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6130812/

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Supplementary Figures:

Figure S1: Selection of study patients with advanced colorectal cancer treated with immune checkpoint inhibitors. Patients were selected from an institutional database of patients with unresectable or metastatic colorectal cancer who underwent genetic sequencing with the MSKCC IMPACT panel.

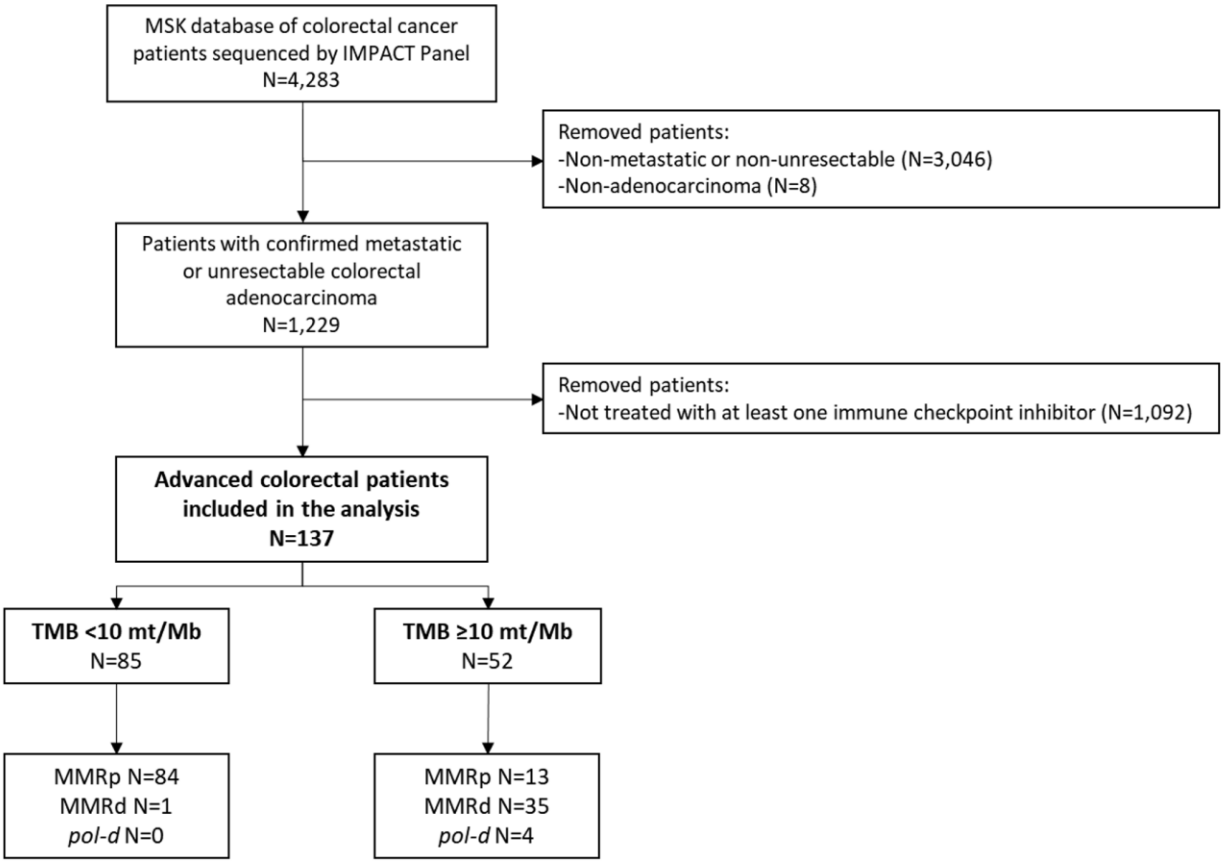


Figure S2: Selection of study patients with advanced multiple malignancies treated with immune checkpoint inhibitors. Patients were selected from an institutional database that compiled patients evaluated in a previous clinical study who had various advanced tumor types all treated with immune checkpoint inhibitors.

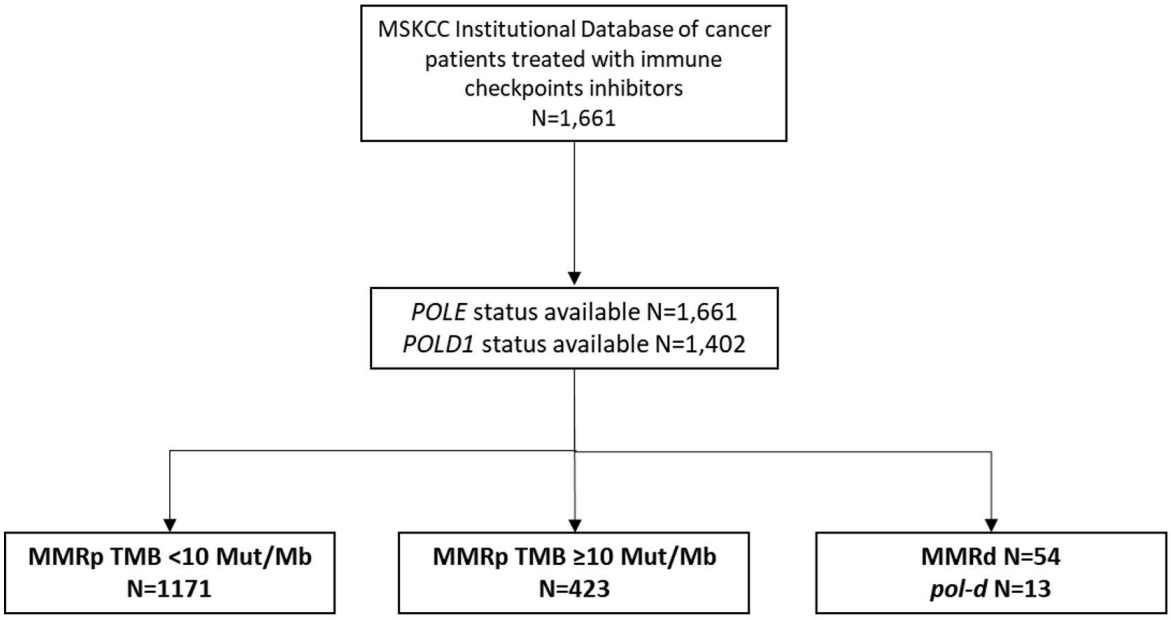
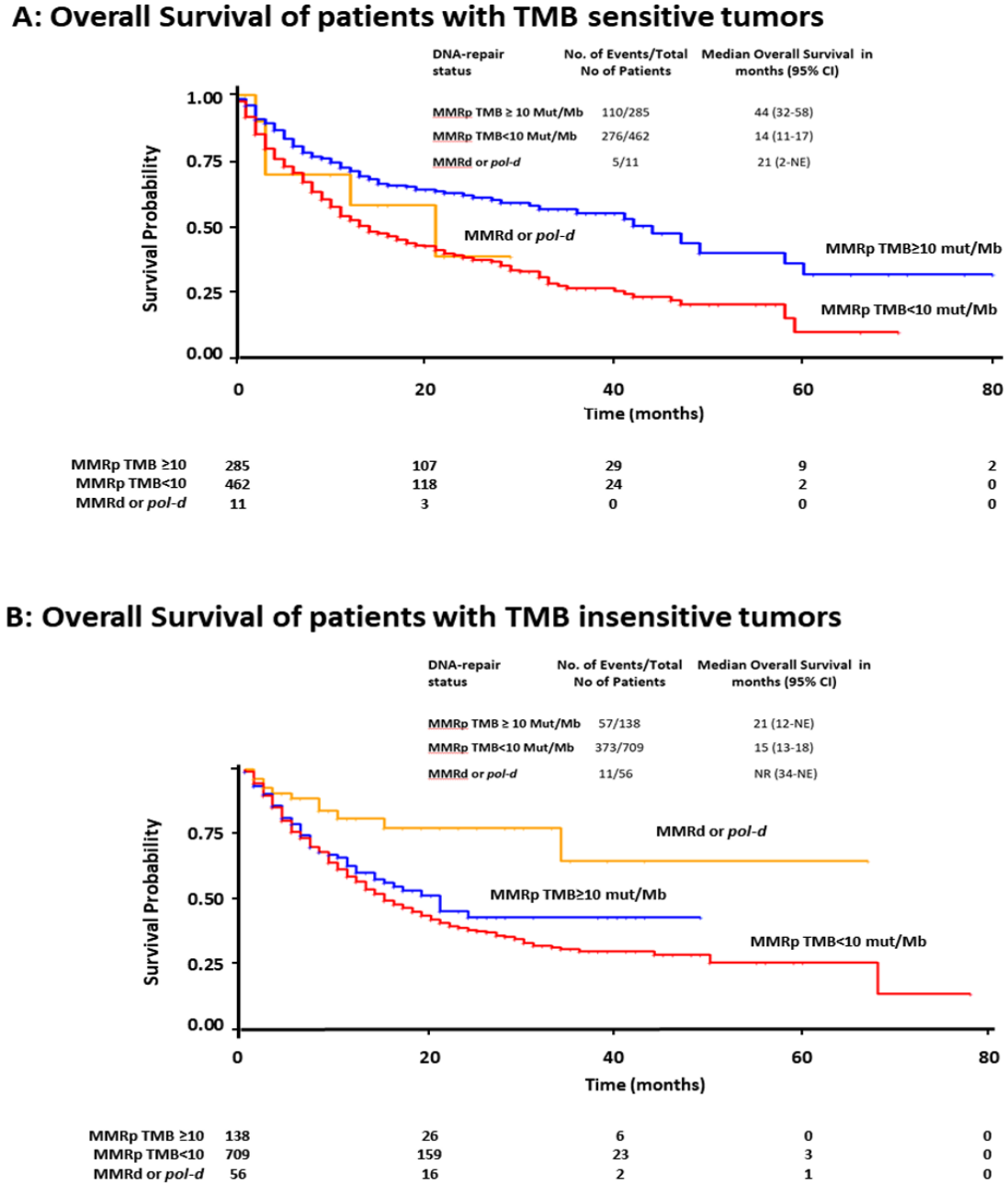


Figure S3: Impact of ICI on survival in validation cohort of patients with multiple tumor types. Kaplan–Meier plots depict overall survival in 1661 patients aggregated into two models based on the presence (TMB sensitive) or absence (TMB insensitive) of a positive association of TMB ≥10 mut/Mb with survival in the respective tumor types. Patients with TMB sensitive tumor types (head and neck, melanoma, or NSCLC) are combined and stratified by TMB and DNA-repair status including MMR-d/*pol-d* status (A). Patients with TMB insensitive tumor types (urinary tract, unknown primary, colorectal, esophagogastric, brain, kidney, breast, uveal melanoma, mucosal melanoma or neuroendocrine tumors) are similarly stratified (B). "NR" indicates not reached and "NE" indicates not evaluable.



Supplemental Table Legends:

Table S1: Clinical and genetic characteristics of the MSKCC colorectal cancer patient cohort according to TMB status. All data was extracted from the electronic medical record with verification of 3% of patient data by licensed physicians. MMR/*pol-d* status classification was assigned per Supplementary methods. Calculations were performed with R statistical software. "NE" indicates not evaluable.

Table S2: Clinical and genetic characteristics of advanced colorectal cancer patients exposed to immune checkpoint inhibitors. All data was manually abstracted from the electronic medical record and verified by licensed physicians. Mutational characteristics are reported from MSKCC IMPACT Next Generation Sequencing panel results. MMR/*pol-d* status classification was assigned per Supplementary Methods. Calculations were performed with R statistical software.

Table S3: Univariate and multivariate analyses of overall survival of advanced colorectal cancer patients exposed to immune checkpoint inhibitors. Survival rates and median overall survival were calculated for all patients with advanced colorectal cancer using Kaplan-Meier analysis. Hazard ratios (HR) for death were calculated using Cox Proportional Hazards Regression. Covariates with HR showing an association (95% CI not overlapping 1.0) between overall survival or progression free survival (Table S6) were placed into a multivariate Cox proportional hazards regression model together. Patients with combination checkpoint inhibitor and non-checkpoint inhibitor therapy were not included in the univariate checkpoint inhibitor treatment analysis. "NR" indicates not reached and "NE" indicates not estimable

Table S4: Hazard ratio for death in cancer patients with tumors treated with immune checkpoint inhibitors classified according to tumor type and stratified by TMB and DNA-repair defect status. Univariate Cox proportional hazards regression was performed to establish HRs for an association between DNA-repair subgroup status and death in each respective tumor type. HRs are represented visually in Figure 1C. An aggregated model was made for patients with breast, kidney, neuroendocrine, uveal melanoma and mucosal melanoma cancers. Patients were aggregated into two models depending on the presence (TMB sensitive) or absence (TMB insensitive) of a positive association of TMB ≥10 mut/Mb with survival in the respective tumor types. "NE" indicates not evaluable

Table S5: TMB and DNA-repair status of patients with multiple cancer types exposed to immune checkpoints inhibitors. Data was obtained from a publicly-available validation cohort of patients with metastatic cancer of different primary tumors treated with immune checkpoint inhibitors from 2015 to 2019. Similar tumor types were consolidated into larger categories according to tumor primary site and pathological results. Mutational and DNA-repair status information was supplemented an internal MSKCC institutional database. *Indicates tumor type combined into "Other Tumors combined" category.

Table S6: Univariate and multivariate analyses of progression-free-survival of advanced colorectal cancer patients exposed to immune checkpoint inhibitors. Survival rates and median progression-free survival were calculated for all patients with advanced colorectal cancer using Kaplan-Meier analysis. Hazard ratios (HR) for death were calculated using Cox Proportional Hazards Regression. Covariates with a positive association between MMRp TMB≥10 status and either progression free

survival or overall survival (HR 95% CI not overlapping 1.0) were placed into a multivariate model together. Patients with combination checkpoint inhibitor and non-checkpoint inhibitor therapy were not included in the univariate checkpoint inhibitor treatment analysis. "NE" indicates non evaluable

Table S7: Survival from time of advanced cancer diagnosis in patients with colorectal cancer treated with or without immune checkpoint inhibitors. Median survival from date of metastatic diagnosis to either documented date of patient death or last follow-up (censored at that moment) was performed using Kaplan-Meier analysis for patients with advanced colorectal cancer who were or were not treated with immune checkpoint inhibitors (ICI). The median time from metastatic diagnosis to date of first ICI treatment was also similarly calculated for patients with advanced CRC who were treated with ICI. "NR" indicates not reached and "NE" indicates not evaluable.

Table S8: Median follow-up time per treatment arm in each study cohort. Median time from immune checkpoint inhibitor treatment to follow-up was performed using Kaplan-Meier analysis for both the advanced annotated colorectal cohort and larger validation cohort of patients with multiple malignancy types.

Table S9: Restricted Mean Survival Time analysis of patients with advanced colorectal cancer treated with ICI. Mean survival time and between-group ratios were determined for patients with advanced colorectal cancer treated with immune checkpoint inhibitors (ICI) using a restricted mean survival time analysis at various time intervals between TMB subgroups (using data represented in Figure 1A) and DNA-Repair Status Subgroups (using data represented in Figure 1B). Between group ratios were determined in relation to the labeled “ref” group.