

Beyond microsatellite testing: assessment of tumor mutational burden identifies subsets of colorectal cancer who may respond to immune checkpoint inhibition

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Background: The clinical application of *PD1/PD-L1* targeting checkpoint inhibitors in colorectal cancer (CRC) has largely focused on a subset of microsatellite instable (MSI-high) patients. However, the proposed genotype that sensitizes these patients to immunotherapy is not captured by MSI status alone. Estimation of tumor mutational burden (TMB) from comprehensive genomic profiling is validated against whole exome sequencing and linked to checkpoint response in metastatic melanoma, urothelial bladder cancer and non-small cell lung carcinoma. We sought to explore the subset of microsatellite stable (MSS) CRC patients with high TMB, and identify the specific genomic signatures associated with this phenotype. Furthermore, we explore the ability to quantify TMB as a potential predictive biomarker of *PD1/PD-L1* therapy in CRC.

Methods: Formalin-fixed, paraffin embedded tissue sections from 6,004 cases of CRC were sequenced with a CLIA-approved CGP assay. MSI and TMB statuses were computationally determined using validated methods. The cutoff for TMB-high was defined according to the lower bound value that satisfied the 90% probability interval based on the TMB distribution across all MSI-High patients.

Results: MSS tumors were observed in 5,702 of 6,004 (95.0%) cases and MSI-H tumors were observed in 302 (5.0%) cases. All but one (99.7%) MSI-H cases were TMB-high (range, 6.3–746.9 mut/Mb) and 5,538 of 5,702 (97.0%) MSS cases were TMB-low (range, 0.0–10.8 mut/Mb). Consequently, 164 of 5,702 (2.9%) MSS cases were confirmed as TMB-high (range, 11.7–707.2 mut/Mb), representing an increase in the target population that may respond to checkpoint inhibitor therapy by 54% (466 vs. 302, respectively). Response to *PD-1* inhibitor is demonstrated in MSS/TMB-high cases.

Conclusions: Concurrent TMB assessment accurately classifies MSI tumors as TMB-high and simultaneously identifies nearly 3% of CRC as MSS/TMB-high. This subgroup may expand the population of CRC who may benefit from immune checkpoint inhibitor based therapeutic approaches.

Keywords: *PD-1*; colorectal cancer; tumor mutational burden; immunotherapy; microsatellite instability

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer globally with estimated 1.4 million new cases and 694,000 deaths in 2012 (1). Contemporary median survivals approaching 30 months in the metastatic setting are seen with fluoropyrimidine-based combinations (FOLFOX, FOLFIRI) (2,3). However, after failure of oxaliplatin and irinotecan containing regimens, prolonged survival is uncommon (4,5).

Immune checkpoint inhibitor response has been observed in a growing number of clinical indications (6). Outside of melanoma, responses are seen in 15–20% of patients treated with single agent *PD-1* or *PD-L1* blocking antibodies across anatomic tumor types (7-9). However, reliable biomarkers capable of predicting response are needed. Increased neo-antigenic burden within tumor cells has been linked to *PD-1/PD-L1* therapeutic response in several indications, however the high cost and significant time associated with neo-antigen discovery/prediction necessitates a more clinically relevant means of predicting response (7,10-12). Microsatellite instability (MSI) status, a genomic signature characterized by deficiencies in the mismatch repair (MMR) proteins *MLH1*, *MSH2*, *MSH6*, and/or *PMS2* and accumulation of short tandem repeating segments of DNA (microsatellites), has emerged as a surrogate for increased tumor mutational burden (TMB). The clinical utility of MSI screening is predicated on identification of microsatellites in the genome of tumor cells either through polymerase chain reaction (PCR), or via immunohistochemical (IHC) staining to determine MMR protein integrity (13,14). Clinical studies have established MSI status as a putative response biomarker for *PD-1* blockade, with progression free survival (PFS) rates of up to 78% reported in MSI-high (MSI-H) colorectal patients, compared to only 11% of microsatellite stable (MSS) patients (11,15). However, the mechanism that drives therapeutic response, increased neo-antigen burden, is only partially characterized by MSI status alone. Recently, evaluation of TMB through next-generation sequencing based comprehensive genomic profiling (CGP) has demonstrated utility in replacing standard MSI screening in CRC patients, with the added benefit of providing additional relevant genomic findings in genes

such as *EGFR*, *KRAS*, *BRAF* and *NRAS* (16,17). Tumor mutational burden derived from CGP may represent a more robust surrogate for predicting response to *PD-1* blockade and can be derived from CGP data. Herein, we explore the feasibility and potential utility of calculating TMB from a next-generation sequencing based CGP panel as a potential predictive biomarker of *PD1/PD-L1* therapy in CRC.

Methods

Formalin-fixed, paraffin embedded tissue sections from 6,004 cases of histologically confirmed CRC were collected from 1,178 unique sites and sequenced using a hybrid capture-based comprehensive genomic profiling (CGP) assay (FoundationOne) (18). Patient demographics were captured and annotated to CGP results, including MSI and TMB status. Approval for this study, including a waiver of informed consent and a HIPAA waiver of authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817).

MSI methods

To determine MSI status using sequencing data generated via a CGP protocol, 114 intronic homopolymer repeat loci with adequate coverage on the CGP panel are analyzed for length variability and compiled into an overall MSI score via principal components analysis (19). Ranges of the MSI score were assigned MSI-high (MSI-H), MSI-ambiguous, or microsatellite stable (MSS) by manual unsupervised clustering of specimens for which MSI status was previously assessed either via IHC if available or approximated by the number of homopolymer indel mutations detected by the FoundationOne assay. This method of determining MSI status was validated for accuracy against currently approved methods, including immunohistochemistry and polymerase chain reaction based assessments, with results demonstrating 95% sensitivity and 98% specificity (n=69). Furthermore, precision of comprehensive genomic profiling based MSI calling was evaluated across 86 replicates spanning MSI-High to MSS status, and determined to be 100% for all evaluated samples (manuscript under review) (19).

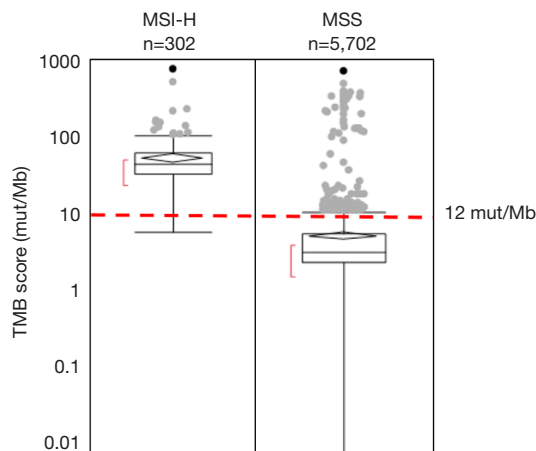


Figure 1 Distribution of TMB across 6,004 advanced colorectal cancer cases stratified by microsatellite instability status. TMB distribution, represented on a log 10 scale, of all 6,004 CRC cases. TMB-high, defined as ≥ 12 mutations/Mb, is represented by the dashed red line. The box plot distributions reveal the overlap of TMB-high amongst both subsets of both MSI-H and MSS cases. The fraction of MSS cases that are TMB-high is 2.9%, or 164 of 5,702 cases. The fraction of MSI-H cases that are TMB-high is 99.7%, or 301 of 302 cases. TMB, tumor mutational burden; CRC, colorectal cancer; MSI-H, microsatellite-high; MSS, microsatellite stable.

TMB methods

TMB was calculated by counting the number of synonymous and non-synonymous mutations across a 1.11 megabase (Mb) region spanning 315 genes, with computational germline status filtering, and reporting the result as mutations/Mb (mut/Mb). This method has been previously validated for accuracy against whole exome sequencing (20). Patients were classified as TMB-high (≥ 11.7 mut/Mb) according to a 90% confidence interval based upon a Weibull distribution of TMB values observed within the MSI-high subgroup. Precision of the TMB values was validated in a separate cohort of 49 patients, replicated 4–6 times each. The TMB value of each patient ranged from 1.8 to 52.2 mut/Mb in the validation cohort. Reproducibility of the TMB status was evaluated against the threshold of 11.7 mutations/Mb used to identify TMB-high for this cohort. Results from the reproducibility evaluation demonstrated that 47 of 49 samples (96%) maintained the same TMB status, and the average coefficient of variation for the TMB score (mut/Mb) was determined to be 15% across all samples (Table S1).

Statistical methods

In the TMB reproducibility study, the average TMB and coefficient of variation was determined from the replicate samples using statistical software provided by Microsoft Excel® (2016 MS Office). Statistical analysis comparing the frequency of somatic variants occurring in either the MSI-H or MSS groups, as well as TMB-high or TMB-low groups within the MSS cohort, was performed using a z-test calculated with JMP software (©SAS Institute, Inc.). Resulting P values were generated to determine significance. In order to identify a cutoff to classify TMB high patients, the distribution of TMB values across all 302 MSI-high patients was fit to a Weibull model, with a goodness of fit test achieving a P value < 0.01 through a Cramer-von Mises W test using JMP software (©SAS Institute, Inc.). The TMB cutoff of ≥ 11.7 mutations/Mb equates to the lower bound of a 90% confidence interval of the expected TMB values associated with MSI-high according to a Weibull fit distribution.

Results

Patient characteristics

A total of 6,004 cases of CRC consisting of 2,817 (46.9%) women and 3,187 (53.1%) men were evaluated from the Foundation Core database between December 2014 and January 2017 across 1,178 medical centers. The median age at the time of tissue biopsy was 55.5 years (range, 8–88 years) in the overall population. Among cases deemed MSI-H, the median age at the time of biopsy was 63.0 years, with 148 female (49.0%) cases and 154 male (51.0%) cases. The median age within the MSS cases was slightly younger at 59 years, with 2,669 (46.8%) female cases and 3,033 (53.2%) male cases.

MSI status and tumor mutational burden

Overall, 5,702 cases (95.0%) were determined to be microsatellite stable (MSS) and 302 cases (5.0%) to be MSI-H by CGP analysis. The reported range of TMB within the total cohort (n=6,004) was 0 to 746.9 mutations/Mb (mut/Mb), with a median of 4.5 mut/Mb (Figure 1). The reported range of all MSI-H cases was 6.3–746.9 mut/Mb, compared to a range of 0–703.6 mut/Mb within the MSS cohort. The median TMB was significantly higher in the MSI-H compared to the MSS cohort (46.8 vs. 3.6 mut/Mb, P value < 0.0001), consistent with the premise that loss of

Table 1 Key genomic features across 6,004 colorectal cancer cases highlighting identification of MSS cases with elevated tumor mutational burden

Variables	MSI-H (%)	MSS (%)	MSS/TMB-high (≥12 mut/Mb) (%)	MSS/TMB-low (<12 mut/Mb) (%)
Total	302	5,702	164	5,538
Median age (years)	63	59	58	59
No. male	154 (51.0)	3,033 (53.2)	90 (54.9)	2,941 (53.1)
No. female	148 (49.0)	2,669 (46.8)	74 (45.1)	2,597 (46.9)
Genomic alterations				
No. <i>BRAF</i>	109 (36.1) ^{***}	453 (7.9)	20 (12.2)	433 (7.8)
No. <i>KRAS</i>	88 (29.1) ^{***}	2,987 (52.4)	76 (46.3)	2911 (52.6)
No. <i>NRAS</i>	6 (2.0)	262 (4.6)	6 (3.7)	256 (4.6)
No. <i>PIK3CA</i>	85 (28.1) ^{***}	1,008 (17.7)	53 (32.3)	955 (17.2)
No. <i>MLH1</i>	56 (18.5) ^{***}	10 (0.2)	4 (2.4)	6 (0.1)
No. <i>MSH2</i>	47 (15.6) ^{***}	20 (0.4)	15 (9.1) ^{###}	5 (0.1)
No. <i>MSH6</i>	85 (28.1) ^{***}	28 (0.5)	12 (7.3) ^{###}	16 (0.3)
No. <i>PMS2</i>	16 (5.3) ^{***}	14 (0.2)	3 (1.8)	11 (0.2)
No. <i>POLE</i>	1 (0.3)	48 (0.8)	34 (20.7) ^{###}	13 (0.2)
No. <i>POLD1</i>	4 (1.3)	5 (0.1)	0 (0.0)	5 (0.1)
No. <i>TP53</i>	100 (33.1) ^{***}	4,414 (77.4)	117 (71.3)	4,297 (77.6)
No. <i>APC</i>	133 (44.0) ^{***}	4,414 (77.4)	122 (74.4)	4,292 (77.5)
No. <i>ERBB2</i>	17 (5.6)	266 (4.7)	7 (4.3)	259 (4.7)
No. <i>EGFR</i>	11 (3.6)	119 (2.1)	8 (4.9)	111 (2.0)

Asterisk designates genes for which alteration frequency differed significantly between MSS and MSI-H samples (***) denotes P value ≤0.001). Number sign designates genes for which alteration frequency differed significantly between TMB-high/MSS and TMB-low/MSS samples (^{###} denotes P value ≤0.001). MSS, microsatellite stable; MSI-H, microsatellite-high; TMB, tumor mutational burden.

function in mismatch repair genes associated with MSI-H status contributes to a higher overall TMB. Nearly all (301/302, 99.7%) MSI-H patients were classified as TMB-high (≥11.7 mut/Mb), and the association of MSI-H status with high TMB was highly statistically significant (P<0.0001). Analysis of the 5,702 MSS CRC cases revealed that 164 (2.9%) were classified as TMB-high (range, 11.7–703.6 mut/Mb) (Figure 1).

Genomic alterations

To further investigate the genomic context of TMB-low/MSS, TMB-high/MSS and MSI-H samples we examined incidence of co-occurring known or likely oncogenic mutations in the genes *NRAS*, *APC*, *TP53*, *PIK3CA*, *BRAF*, *KRAS* and *EGFR* (Table 1). Additionally, we recorded the

frequency of patients harboring a known or likely alteration in the MMR and DNA proof reading genes *MLH1*, *MSH2*, *MHS6*, *PMS2*, *POLE* and *POLD1*. Comparison between MSI-H and MSS cases, regardless of TMB score, demonstrated that *BRAF*, *EGFR*, *PIK3CA* or *ERBB2* variants occurred more frequently in the MSI-H cohort, while variants in *TP53*, *NRAS*, *KRAS*, or *APC* were observed less frequently, respectively (Table 1). As anticipated, patient samples with at least one known or likely driver variant in the MMR genes *MLH1*, *MSH6*, *MSH2* or *PMS2* were highly enriched in the MSI-H population compared to the MSS population (18.5% vs. 0.2%; 28.1% vs. 0.5%, 15.6% vs. 0.4%, 5.3% vs. 0.2%, respectively; P value <0.0001). Known or likely driver events in the proofreading gene *POLD1* were more frequently observed in the MSI-H cohort (1.3% vs. 0.1%, respectively; P value =0.0604).

Variants within *POLE* were more frequently observed in the MSS cohort (0.8% vs. 0.3%, respectively; P value =0.1230) (Table 1).

Evaluation of the TMB-high vs. TMB-low groups within the MSS cohort revealed that TMB-high/MSS patients were approximately 100× more likely to harbor known and likely variants in *MSH2* (9.1% vs. 0.1%, respectively; P value <0.0001) and *POLE* (20.7% vs. 0.2%, P value <0.0001) compared to TMB-low/MSS patients. Among all of the *POLE* variants found in the TMB-high/MSS cohort, P286R, V411L and A456P accounted for 51% of the total. Variants in *MSH6* (7.3% vs. 0.3%; P value =0.0537) and *MLH1* (2.4% vs. 0.1%; P value <0.0001) were approximately 20× more frequent in TMB-high/MSS vs. TMB-low/MSS patients, and variants in *PMS2* (1.8% vs. 0.2%; P value =0.1201) occurred about 9× more frequently in TMB-high/MSS vs. TMB-low/MSS patients. Variants in *POLD1* were not observed with significant frequency (<0.1%) in either TMB-high or TMB-low patients within the MSS cohort. Other driver genes were found at less than 3× differences between the TMB-high/MSS and TMB-low/MSS groups (Table 1).

Illustrative case 1

A 35-year-old female was diagnosed with stage IV rectal adenocarcinoma after presenting with rectal and buttock pain. Her tumor was *KRAS* wild type by hotspot PCR covering codons 12/13/61, MSS (by IHC), and germline negative for *MLH1*, *MSH2*, *MSH6* and *PMS2* alterations during testing for Lynch syndrome. She progressed on FOLFOX and panitumumab and second line FOLFIRI with worsening hepatic metastases and pelvic pain (Figure 2). To guide treatment options a biopsy was subjected for CGP (FoundationOne) that confirmed MSS CRC, but revealed a highly elevated TMB (223 mutations/Mb), and mutations in *KRAS* (at codon 146), *MSH2*, *PIK3CA*, *PMS2*, *BRCA2*, *PIK3CA*, *POLE*, and *TP53*. Given her refractoriness to chemotherapy, poor performance status, and emerging data supporting benefit of *PD-1* blockade in TMB-high tumors, she was treated off-label with pembrolizumab. She had rapid symptomatic improvement and a significant radiographic response at 12 weeks and continues to benefit, now 7 months since starting therapy (Figure 2).

Illustrative case 2

A 45-year-old Caucasian female was diagnosed with stage III

rectal cancer, MSS by initial immunohistochemical analysis. Following initial therapy, she developed pelvic sidewall and biopsy-proven anastomotic recurrence but declined salvage surgical options or chemoradiotherapy. To explore maximal options her original surgical sample was sent for CGP, revealing a MSS, *RAS/RAF* wild type tumor with high TMB of 14 mut/Mb. Known alterations in *APC* and *TP53* were additionally identified. She was started on off-label nivolumab and achieved a complete response, now lasting over 18 months with no endoscopic evidence of tumor.

Discussion

Herein, we report analysis from a large cohort of over 6,000 colorectal cancer patients and describe a TMB threshold that identifies 99.7% of MSI-H patients, while capturing an additional 3% of MSS samples, increasing the potential treatment population by 54%. Our series suggests TMB and MSI can be reliably derived from CGP data, and can identify an additional cohort of patients (MSS/TMB-high) who may benefit from immune checkpoint inhibitors.

Immuno-oncology studies have highlighted several potential biomarkers of varying large scale clinical feasibility including *PD-1/L1* IHC, tumor infiltrating lymphocytes (TILs), and elevated numbers of nonsynonymous mutations (21-25). Elevated *in-silico* predicted class I neoantigen load is emerging as a robust predictive response biomarker to checkpoint inhibitor therapies, but has been derived from whole-exome sequencing (WES), a time and cost intensive method not widely available (10). Our series identifies the TMB distribution across MSI-H colorectal cancers, but more importantly suggests that nearly 1 in every 33 MSS colorectal patients have an elevated mutational burden according to a classification based upon MSI status. While MSI-H tumors are seen in 12–22% of stage II and III CRC respectively, MSI-H is observed in only 3–5% of stage IV patients (26,27). Thus, among the roughly 50,000 CRC-related deaths per year in the US, CGP has the potential to identify 1,500 patients/year with MSS/TMB-high tumors (28).

Interestingly, the CGP-derived genomic features associated with the MSS/TMB-high cohort are suggestive of a mismatch repair defective state that likely induces increased TMB through spontaneous *POLE* loss of function (Table 1). The implication of an enriched *POLE* genotype within the MSS/TMB-high group supports the hypothesis that defects in both the MMR and DNA proofreading pathway can cause a hypermutated state, without necessarily giving rise to the short tandem repeat signature observed

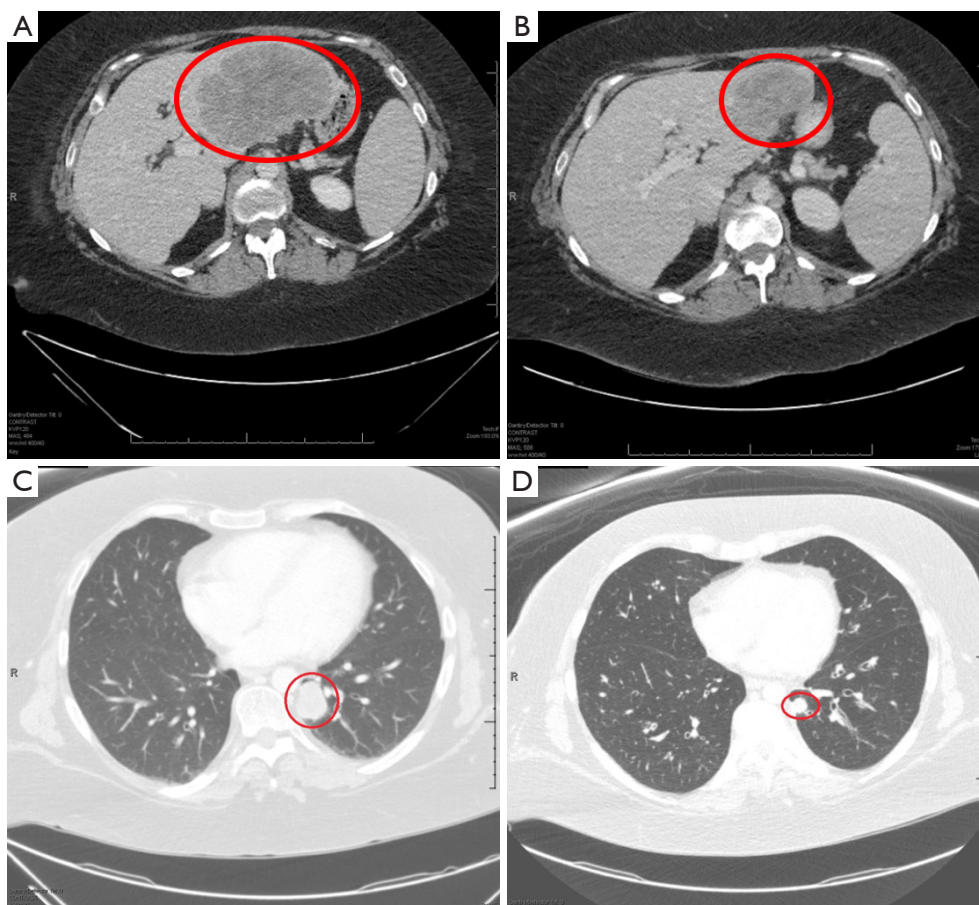


Figure 2 Microsatellite stable CRC case 1 with TMB-high (223 mutations/Mb) demonstrating response to immunotherapy. Response to anti-*PD-1* monotherapy in a patient with MSS advanced colorectal cancer that was found to be TMB-high (223 mutations/Mb). Partial response after 4 cycles of pembrolizumab is highlighted by 50% reduction in hepatic metastatic disease (A vs. B) and significant improvement in pulmonary metastasis (C vs. D). CRC, colorectal cancer; TMB, tumor mutational burden; MSS, microsatellite stable.

through classic MSI-H testing. Our first patient case harbored the classic genomic features associated with the MSS/TMB-high group (*MSH2+*, *POLE+*). Pathogenic *POLE* aberrations have been seen to identify patients responding to *PD-1* inhibitors in endometrial cancers (29-31). Case 2 highlights the observation that perhaps even a slightly elevated TMB increases the likelihood of an immunogenic neo-epitope to drive an immune response, as in theory only a single neo-epitope may be needed.

Recently, CGP-derived TMB (by the same assay used here) was identified as an independent predictive response marker in trials evaluating various checkpoint inhibitors and combinations in bladder cancer, NSCLC and metastatic melanoma (7,32,33). Further work is needed to determine if increasing TMB has a linear relationship with probability of

response to immunotherapies, but our work suggests CGP can reliably determine TMB over a dynamic range reflective of patients seen in clinical practice. Notably, additional MSS/TMB-high cases (n=2) with exceptional responses to checkpoint inhibitor therapy are recently described, adding further support to our findings (34,35). Beyond TMB, it is important to note that MSI determination by NGS is not considered standard, however, mounting published data suggests excellent agreement with standard IHC and PCR methods (manuscript under review) (18).

The rates of MSI-H samples (5.0%) in our series is in agreement with the frequencies recorded from clinical trials in advanced CRC, suggesting our cohort accurately reflects the advanced CRC landscape (26). Similarly, *KRAS* and *BRAF* frequencies support this, however, we acknowledge a

potential selection bias as samples subjected to the CGP assay (FoundationOne) used in our series may have been previously tested by orthogonal means. Additionally, we cannot draw conclusions about the responsiveness to immune-mediated therapies of MSS/TMB-high patients on the lower end of the TMB spectrum based on anecdotal evidence alone. While it can be argued that our first case harbors genomic alterations known to be associated with checkpoint inhibitor response it is important to note that neither the *POLE* mutation or *PD-L1* and *PD-L2* gene amplification would have been detected by standard of care testing in CRC.

In the trial by Le and colleagues using traditional MSI testing (Promega MSI Assay, Promega Corporation) no responses were observed in MSS colorectal cancers (15). However, the sample size of patients with MMR-proficient tumors (defined by MSI negative) was only 18, and not likely to have included an MSS/TMB-high tumor given the 3% incidence rate. Prospective knowledge of MSS/TMB-high status may well have resulted in responses to anti-*PD-1* as suggested herein. To our knowledge, our study represents the largest series in CRC investigating TMB using a validated CGP assay, and identifies a 3% frequency of high mutational burden in MSS tumors. Further prospective study of MSS/TMB-high patients is warranted.

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Footnote

Conflicts of Interest: DA Fabrizio, G Frampton, J Sun, K Gowen, M Kennedy, J Greenbowe, AB Schrock, JS Ross, PJ Stephens, SM Ali, and VA Miller are employees and hold equity in Foundation Medicine, Inc. DA Fabrizio holds equity in Juno Therapeutics and Seattle Genetics. SJ Klempner has received honoraria from Foundation Medicine, Inc. The other authors have no conflicts of interest to declare.

Ethical Statement: Approval for this study, including a waiver of informed consent and a HIPAA waiver of authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817).

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Supplementary**Table S1** Reproducibility of tumor mutational burden across the training cohort of 49 patient samples subjected to comprehensive genomic profiling. The average coefficient of variation across the groups was 14.7% and the reproducibility according to the cutoff of 11.7 mut/Mb is 96%

Replicate number	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Replicate 1	52.2	37.8	11.7	5.4	5.4	3.6	1.8	2.7	2.7
Replicate 2	50.4	40.5	9.9	8.1	7.2	3.6	5.4	3.6	1.8
Replicate 3	52.2	39.6	13.5	9.0	6.3	3.6	2.7	3.6	1.8
Replicate 4	48.6	40.5	10.8	7.2	7.2	4.5	3.6	3.6	1.8
Replicate 5	n/d	37.8	10.8	6.3	7.2	3.6	3.6	2.7	1.8
Replicate 6	n/d	n/d	10.8	6.3	7.2	3.6	n/d	2.7	n/d
Average TMB	50.8	39.2	11.2	7.0	6.7	3.7	3.4	3.1	2.0
Standard deviation	1.7	1.4	1.2	1.3	0.8	0.4	1.3	0.5	0.4
CV (%)	3.4	3.5	11.0	18.8	11.2	9.8	39.0	15.5	20.3

n/d denotes value not determined for lack of sample availability. TMB, tumor mutational burden.