# Role of Tumor Mutation Burden Analysis in Detecting Lynch Syndrome in Precision Medicine: Analysis of 2,501 Japanese Cancer Patients

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## ABSTRACT

**Background:** Tumor mutation burden (TMB) is the total exonic mutation count per megabase of tumor DNA. Recent advances in precision medicine occasionally detect Lynch syndrome (LS) by germline sequencing for mismatch-repair (g.*MMR*) genes but not using TMB. The current study analyzes the utility of TMB in detecting LS.

**Methods:** Whole-exome sequencing (ion-semiconductor sequencing) was performed for somatic and germline DNA from 2,501 various cancer patients to detect TMB and g.*MMR* sequencing. MMR IHC was conducted when high TMB (≥10) was detected in LS-related cancers with an additional condition of wild-type *BRAF* in colorectal cancers. Target sequencing and multiplex ligation-dependent probe amplification (MLPA) were further performed for g.*MMR* genes in MMR-deficient cancers (TMB-based g.*MMR* target sequencing). We compared universal sequencing and TMB-based target sequencing in their sensitivity for detecting LS.

**Results:** LS was detected in 16 (0.6%) of the 2,501 patients: 1.1% (9/826) of colorectal cancer patients, 16.2% (6/37) of endometrial cancer patients, and 14.3% (1/7) of small intestine cancer patients. TMB-based g.*MMR* target sequencing (81.3%) showed superior sensitivity for detecting LS than universal g.*MMR* sequencing (56.3%; $P = 0.127$) but missed 3 LS patients (1 with a low-TMB cancer, 1 with a *BRAF*-mutant colorectal cancer, and 1 with an MMR-proficient cancer). Ion-semiconductor sequencing could detect single-nucleotide substitutions but not large deletions. *POL*-mutated cancers showed extremely high TMBs (48.4–749.2).

**Conclusions:** g.*MMR* target sequencing, combined with TMB, somatic *BRAF* mutation, and MMR IHC is an effective strategy for detecting LS.

**Impact:** TMB can be a biomarker for detecting LS in precision medicine.

## Introduction

Tumor mutation burden (TMB) is defined as the total count of single-nucleotide variations, insertions, and deletions (indels) per each megabase (Mb) in the coding region of tumor DNA and is also a predictive biomarker for the response to immune-checkpoint inhibitors (1–3). Today, next-generation sequencing (4) is being increasingly applied in the oncological field to genetically characterize tumors and select the most suitable treatment agent or "precision medicine." TMB can be measured not only through whole-exome sequencing (WES) but also by multigene panel testing using NGS on fresh-frozen (5) or formalin-fixed paraffin-embedded (FFPE) cancer tissues (6). TMB degree varies among human cancers, tending to be higher in the type of lung cancer developed in smokers, cutaneous squamous carcinoma, melanoma, and colorectal carcinoma; in contrast, it is lower in non–small cell-type lung cancer, breast cancer, pancreatic cancer, and childhood cancers (6–8). TMB is also high in cancers associated with microsatellite instability (MSI), which is caused by the promoter methylation of *MLH1* (often coupled with *BRAF* V600E mutation in colorectal cancer; refs. 4, 9) and by mutations of DNA mismatch-repair (*MMR*) genes (6). TMB is further higher in cancers exhibiting somatic and germline mutation of DNA polymerase proofreading genes, including *POLE* and *POLD1* (10–12).

Lynch syndrome (LS) is an autosomal dominant cancer predisposition syndrome caused by germline variants in *MMR* genes (*MLH1, MSH2, MSH6*, and *PMS2*), accounting for 1% to 4% of colorectal cancer (13–16) and 2% to 6% of endometrial cancers (EC; refs. 17–19). Variant carriers are at risk of early-onset colorectal cancer (15, 16), EC (17, 18), upper tract urothelial cancers (20, 21), and a spectrum of other associated tumors (22), and, therefore, the detection of g.*MMR* variant carriers is crucial in reducing the number of cancer-related deaths via the surveillance of target organs.

The standard diagnosis of LS begins with the selection of high-risk individuals by reviewing their and their family's cancer history based on the Amsterdam II criteria or revised Bethesda guidelines (14). These patients are then further evaluated by the IHC of MMR proteins as well as MSI analysis in their tumors. A final diagnosis is made based on deleterious g.*MMR* gene variants detectable via germline DNA sequencing in MSI-high and/or MMR-deficient cancer patients (13, 14). However, these guidelines and criteria were

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established only for colorectal cancer patients, with limited sensitivity for LS. Therefore, recently, the strategy for LS detection has shifted toward universal screening using MMR IHC and/or MSI analysis for all or for age-specific conditions of LS-related cancers (18, 19, 23–25). However, to date, TMB has not been used as a marker for screening LS.

This study analyzed the utility of TMB analysis using WES to detect LS in cancer patients, especially LS-associated cancer patients.

## Materials and Methods

### Patients and samples

In January 2014, the Shizuoka Cancer Center started a project with high-tech omics-based patient evaluation (Project HOPE) that performed genome-wide exome sequencing in germline and somatic DNAs obtained from various cancer patients (26). From January of 2014 through June of 2016, 2,501 cancer patients who agreed on this study and had a tumor large enough for the genomic analysis participated in the current study, within total 8,549 cancer patients who underwent surgical resection for their cancers in the Shizuoka Cancer Center (**Table 1**). Hence, almost all participants presented with good performance status (PS: 0–1) and had cancers in the resectable stage. All cancers were pathologically confirmed using surgical samples.

At the initial hospital visit, patients and their families filled out questionnaires concerning disease history, family history, and lifestyle aspects. The nurses reconfirmed the content of the questionnaires by conducting interviews for about 20 to 30 minutes with each patient. LS-associated cancers were defined to be limited to colorectal cancer, EC, renal pelvic and ureteral cancers, and small intestine cancer (13). The conditions of the revised Bethesda guidelines (14) were evaluated in cases with colorectal cancer. None of the patients or their families had been diagnosed with LS before study entry. The Institutional Review Board of the Shizuoka Cancer Center ethically approved this study (IRB no. 2019-24), and all procedures were conducted in accordance with the Helsinki Declaration. Written informed consent for this study was obtained from all participants prior to study entry.

### WES of somatic and germline DNA

In this study, WES was performed to analyze somatic and germline DNA (27). Somatic DNA analyses included TMB, total exonic mutation numbers, mutations of *BRAF*, 4 *MMR* genes, and DNA polymerase proofreading genes (*POLE* and *POLD1*). Germline DNA analyses were limited to g.*MMR* genes to detect pathogenic variants and diagnose LS. A pair of DNA samples extracted from blood and surgically resected fresh-frozen cancer tissues were collected at each surgery and stored at −70°C. Fresh surgical specimens were diagnosed by a pathologist, and each tumor was histologically estimated to weigh >100 mg and contain >50% concentration tumor cells. DNA was extracted using a QIAmp Kit (QIAGEN) according to the manufacturer's instructions, with a process of proteinase K addition. WES was performed with 100 ng of template DNA using an Ion Torrent AmpliSeq Exome RDY Panel kit (Thermo Fisher Scientific) and the Ion Torrent Proton semiconductor DNA sequencer (Thermo Fisher Scientific) following the manufacturer's recommended protocol as written previously (5, 26, 27). The threshold of WES was set to 30%,

**Table 1.** Cancer types and demographics of 2,501 cancer cases.

| Cancer origin/type | Number of cases | | Gender (*n*) | | Age (y.o.) |
|---|---|---|---|---|---|
| | Resected cases[a] | Cases in this study | Male | Female | Mean ± SD |
| Colon | 1,347 | 826 | 502 | 324 | 66.3 ± 11.9 |
| Lung | 760 | 420 | 247 | 173 | 69.4 ± 8.9 |
| Stomach | 936 | 283 | 206 | 77 | 69.7 ± 10.5 |
| Head and neck | 868 | 195 | 143 | 52 | 64.4 ± 12.8 |
| Breast | 957 | 181 | 1 | 180 | 57.7 ± 12.9 |
| Liver | 333 | 112 | 88 | 24 | 69.5 ± 9.4 |
| Pancreas[b] | 215 | 60 | 34 | 26 | 71.3 ± 8.6 |
| Skin | 460 | 42 | 23 | 19 | 61.5 ± 21.8 |
| Brain | 191 | 40 | 24 | 16 | 56.7 ± 15.0 |
| Ovary | 311 | 37 | 0 | 37 | 59.3 ± 13.7 |
| Endometrium | 239 | 37 | 0 | 37 | 61.8 ± 12.2 |
| Biliary tract | 160 | 30 | 20 | 10 | 69.0 ± 6.9 |
| Esophagus | 118 | 27 | 23 | 4 | 67.9 ± 8.9 |
| Kidney | 195 | 27 | 11 | 16 | 62.7 ± 14.1 |
| Soft tissue | 206 | 24 | 17 | 7 | 58.3 ± 22.1 |
| Uterus cervix | 413 | 15 | 0 | 15 | 51.3 ± 15.9 |
| Bone | 104 | 7 | 3 | 4 | 29.9 ± 21.6 |
| Small intestine | 52 | 7 | 5 | 2 | 64.0 ± 13.5 |
| GIST[c] | 42 | 38 | 22 | 16 | 63.4 ± 12.7 |
| NET/NEC[d] | 41 | 31 | 19 | 12 | 64.7 ± 10.9 |
| Lymphoma[e] | 3 | 3 | 2 | 1 | 62.7 ± 9.1 |
| Others | 598 | 59 | 27 | 32 | 55.3 ± 17.1 |
| Total | 8,549 | 2,501 | 1,417 | 1,084 | 65.7 ± 12.7 |

[a]Total number of surgically resected cases during the study period.
[b]Pancreas including ductal adenocarcinoma and intraductal papillary neoplasm (IPMC: 5, IPMA: 1).
[c]Stomach: 22, colon: 5, small intestine: 9, esophagus: 1, omentum: 1.
[d]Lung: 10, pancreas: 10, small intestine: 3, liver: 3, head and neck: 1, esophagus: 1, uterus cervix: 1, biliary tract: 1, and colon: 1.
[e]Colon: 1, brain: 1, and bone: 1.

and <30% of an allele ratio was not judged to be germline variants (28). Loss of heterozygosity (29) was analyzed in the LS-associated cancers with high TMB (hTMB; ≥10 mutations/Mb; refs. 10, 30). LOH analysis was performed using somatic copy-number alteration analysis using sequencing and SNP array data (SAAS-CNV; ref. 31). Pathogenicity of the detected variant was judged based on the consensus recommendation of American College of Medical Genetics (32). Variant of single-nucleotide substitution or small insertion/deletion was judged by using the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/). Null variants (nonsense, frameshift, canonical ± 1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene, where loss of function is a known mechanism of disease, were basically judged as pathogenic (32), also refereeing to the general population data (minor allele frequency ≥0.5% was judged as nonpathogenic), computational and predictive data, functional data, and so on.

### Comparison of two approaches for detecting germline *MMR* variants

We compared the detection rates of LS (g.*MMR* variant carrier) of universal g.*MMR* gene sequencing using the above-mentioned ion-semiconductor sequencing (universal g.*MMR* sequencing approach; ref. 27) and g.*MMR* target sequencing coupled with multiplex ligation-dependent probe amplification (MLPA; hTMB-based g.*MMR* target sequencing approach). The latter approach was implemented in the cases of LS-related cancers (13) with high levels of TMB, MMR deficiency (dMMR) based on IHC, and an additional condition of negative somatic *BRAF* V600E mutation in cases of colorectal cancer.

For the hTMB-based g.*MMR* target sequencing approach, we defined a tumor with 10 mutations per Mb as hTMB, as per The Cancer Genome Atlas (TCGA; ref. 10). MMR IHC or MSI were recommended for all colorectal cancer patients with hTMB and without somatic *BRAF* mutation and were implemented when written informed consent was obtained. When either MMR IHC or MSI was revealed to be abnormal, target sequencing and MLPA for g.*MMR* genes were additionally examined after obtaining additional informed consent. For g.*MMR* target sequencing, genomic DNA was isolated from peripheral blood via a QIAamp DNA Blood Kit (QIAGEN). DNA was then amplified via PCR using oligonucleotide primers for *MLH1, MSH2, MSH6*, and *PMS2*, and then a library was prepared using the Nextera XT System (Illumina, Inc.). Samples were pooled for multiplexed sequencing, which was performed on MiSeq (Illumina) by a 150-bp paired-end sequence using the MiSeq Reagent Kit v2 (Illumina). Sequenced reads were mapped against reference sequences, and variants were identified by Genomics Workbench Software v7.5 (QIAGEN) and confirmed by Omixon Target Software (Omixon). All variants, except for polymorphism, detected by g.*MMR* target sequencing were again confirmed via Sanger sequencing. MLPA analysis was performed using the Salsa P003 kit for the *MLH1* and *MSH2* genes (MRC-Holland) and was performed according to the manufacturer's instructions. Amplification products were electrophoresed on the ABI 3130xl genetic analyzer (Applied Biosystems). Data analysis for detecting copy-number variations was performed using Coffalyser.net software (MRC-Holland).

### IHC of the MMR protein

Thin-sliced sections (3-μm thickness) of FFPE cancer tissues from the surgical specimen were prepared for the evaluation of MMR protein expression (27). After deparaffinization with xylene for 15 minutes and stepwise de-xylene treatment with ethanol, heat treatment was applied using Epitope Retrieval Solution 2 (Leica Biosystems) at 95°C for 20 minutes to activate antigenicity. As a primary antibody against each MMR protein, the anti-hMLH1 antibody (Clone ES05, ×50 dilution, Dako), anti-hMSH2 antibody (Clone FE11, ×50 dilution, Dako), anti-hMSH6 antibody (Clone EP49, ×50 dilution, Dako), and anti-hPMS2 antibody (Clone EP51, ×25 dilution, Dako) were used. The secondary antibody was reacted at room temperature for 8 minutes using Bond Polymer Refine Detection (Leica Biosystems). Then, color was developed using diaminobenzidine (SIGMA) for 10 minutes at room temperature.

When the MMR protein was not expressed or severely repressed in the cancer tissue, contrasting the diffuse expression in the nonneoplastic tissue, the tumor was determined to have a negative MMR expression. This histologic evaluation was conducted by the expert pathologist (T. Oishi).

### MSI analysis

MSI was analyzed after the acquisition of a separate written informed consent for the diagnosis of LS (27). MSI analysis was entrusted to Falco Holdings Co., Ltd. and performed using the MSI Analysis System, Version 1.2 (Promega Corporation) following the manufacturer's recommended protocol. MSI status was evaluated by comparing normal and tumor DNAs using 5 nearly monomorphic mononucleotide microsatellite markers (BAT25, BAT26, NR21, NR24, and MONO27). A high frequency of microsatellite instability (MSI-H) was defined when the tumor DNA demonstrated instability in 2 or more markers, whereas a low frequency of MSI (MSI-L) was defined when only one marker was instable and microsatellite stability when a null marker showed instability.

### Gene-expression profiling

Gene-expression profiling was performed to compare the *MLH1* gene expressions of *BRAF* V600E wild-type and *BRAF* V600E-mutant-hTMB colorectal cancers. Gene-expression profiling was analyzed by following the previously described protocol (33).

### Statistical analysis

Statistical analyses were performed using JMP ver.12.2.0 statistical software (SAS Institute Japan Ltd.). A Fisher exact test or $\chi^2$ test was used to assess the categorical variables. Student *t* test or Mann–Whitney *U* test were used to analyze the continuous variables. A probability value <0.05 was considered statistically significant.

## Results

### TMB in various human cancers

Depth of coverage in the current WES for somatic DNA was 121.7 ± 850.9 (average ± SD) by ion-proton semiconductor sequencing. TMB values and hTMB ratios by cancer type are shown in **Table 2**. The average TMB levels were highest in ECs (65.3 ± 155.0) followed by skin cancers (13.4 ± 39.4), small intestine cancers (8.9 ± 14.8), colorectal cancers (7.8 ± 24.2), and gastric cancers (7.2 ± 14.0). Meanwhile, the ratio of hTMB (≥ 10 mutations/Mb) was highest in ECs (40.5%), followed by small intestine cancers (28.6%), skin cancers (16.7%), gastric cancers (14.5%), and lung cancers (11.7%). The average TMB of LS-associated cancers (10.3 ± 41.0) was significantly higher than that in other cancer types (4.0 ± 10.0; $P < 0.0001$). The TMB of lung cancers (4.3 ± 7.6) and neuroendocrine tumor/carcinomas (3.6 ± 4.1) was lower than the average of total cancers; nevertheless, the hTMB ratio was at a high level in these cancers (11.7% and 12.9%, respectively).

**Table 2.** TMB score and rate of hTMB in 2,501 cancer cases.

| Cancer type | *n* | TMB (mean ± SD) | *P* value | Ratio of hTMB[a] % (*n*) | | *P* value |
|---|---|---|---|---|---|---|
| Lynch syndrome–associated cancer types | | | | | | |
| Colon | 826 | 7.8 ± 24.2 | | 7.0% (58) | | |
| Endometrium | 37 | 65.3 ± 155.0 | 10.3 ± 41.0B | 40.5% (15) | 8.6%D | |
| Small intestine | 7 | 8.9 ± 14.8 | | 28.6% (2) | | |
| Ureter | 0 | — | | — | | |
| Other cancer types | | | | | | |
| Lung | 420 | 4.3 ± 7.6 | | 11.7% (49) | | |
| Stomach | 283 | 7.2 ± 14.0 | | 14.5% (41) | | |
| Head and neck | 195 | 3.2 ± 4.2 | | 4.1% (8) | | |
| Breast | 181 | 1.5 ± 1.8 | | 0.6% (1) | | |
| Liver | 112 | 4.2 ± 5.4 | | 1.8% (2) | | |
| Pancreas | 60 | 0.5 ± 0.7 | | 0 | | |
| Skin | 42 | 13.4 ± 39.4 | <0.0001B, C | 16.7% (7) | | 0.145D, E |
| Brain | 40 | 1.5 ± 0.9 | | 0 | | |
| Ovary | 37 | 2.5 ± 5.1 | 4.0 ± 10.0C | 2.7% (1) | 7.0%E | |
| Biliary tract | 30 | 2.2 ± 2.1 | | 0 | | |
| Esophagus | 27 | 2.6 ± 1.5 | | 0 | | |
| Kidney | 27 | 1.6 ± 0.7 | | 0 | | |
| Soft tissue | 24 | 1.3 ± 1.4 | | 0 | | |
| Uterus cervix | 15 | 3.2 ± 4.3 | | 7.7% (1) | | |
| Bone | 7 | 1.2 ± 1.1 | | 0 | | |
| GIST | 38 | 0.7 ± 0.3 | | 0 | | |
| NET/NEC | 31 | 3.6 ± 4.1 | | 12.9% (4) | | |
| Lymphoma | 3 | 4.6 ± 2.9 | | 0 | | |
| Others | 59 | 0.8 ± 0.9 | | 0 | | |
| Total | 2,501 | 6.2 ± 25.7 | | 7.6% (189) | | |

[a]hTMB is defined as >10 of TMB in tumor DNA.

### Demographics of patients with colorectal cancer and EC with hTMB

Patient demographics were compared between the hTMB and low-TMB (lTMB) groups for patients with 2 LS-associated cancer types, colorectal cancer (**Table 3**) and EC (**Table 4**), as the current study analyzed only one patient with hTMB small intestine cancer and null patients with renal pelvis or ureter cancer. hTMB cancer patients accounted for 7.0% of the 826 colorectal cancer patients and 40.5% of the 37 EC patients, and their incidences were significantly different ($P < 0.0001$). Average age of the hTMB cancer patients was almost equal to that of the lTMB cancer patients with colorectal cancer (65.1 vs. 66.1 years; **Table 3**), but the same comparison was nearly significant in patients with EC (57.5 vs. 64.7 years, $P = 0.076$; **Table 4**). The incidence of the patients meeting the revised Bethesda criteria was significantly higher in the hTMB colorectal cancer group (34.5%) than in the lTMB group (21.7%; $P = 0.024$). Also, in the ECs, the incidence of either a

**Table 3.** Demographics and tumor findings in colorectal cancer cases based on the level of TMB.

| | | hTMB group (*n* = 58) | lTMB group (*n* = 768) | *P* value |
|---|---|---|---|---|
| Patient findings | | | | |
| Proportion | | 7.0% | 93.0% | |
| Gender (male/female) | | 30/28 | 472/296 | 0.143 |
| Age (y.o.) | | 65.1 ± 15.5 | 66.1 ± 11.6 | 0.537 |
| Brinkman index[a] | | 384.9 ± 521.2 | 398.1 ± 481.6 | 0.841 |
| Rev. Bethesda criteria[b] | | 34.5% | 21.6% | 0.024 |
| Tumor findings | | | | |
| TMB (/mb) | | 67.6 ± 67.3 | 3.3 ± 1.4 | <0.0001 |
| Number of mutation[c] | | 2,221.8 ± 2,194.3 | 108.3 ± 44.9 | <0.0001 |
| POLE or POLD1 exonuclease domain mutant | | 17.2% (10/58) | 0% (0/768) | <0.0001 |
| Average MLH1 expression | *BRAF V600E mutant* | −2.0 ± 0.8 | — | <0.0001 |
| | *BRAF V600E wild* | −0.1 ± 0.8 | — | |
| MSI | | 66.7% (22/33) | NA | — |
| dMMR | | 69.7% (23/33) | NA | — |

[a]Brinkman index = number of cigarettes smoked/day × number of years smoked.
[b]Incidence of the patients meeting the revised Bethesda criteria; dMMR: mismatch-repair deficiency.
[c]Mutation number within the coding region of tumor DNA.

**Table 4.** Demographics and tumor findings in endometrial cancer cases based on the level of TMB.

| | hTMB group ($n = 15$) | lTMB group ($n = 22$) | *P* value |
|---|---|---|---|
| Patient findings | | | |
| Proportion | 40.5% | 59.5% | |
| Age (y.o) | 57.5 ± 13.3 | 64.7 ± 10.8 | 0.076 |
| Brinkman index[a] | 19.3 ± 64.6 | 26.8 ± 63.1 | 0.728 |
| Incidence of personal or family history of LS-associated cancer (*n*) | 40.0% (6) | 18.2% (4) | 0.142 |
| Tumor findings | | | |
| TMB (/mb) | 157.7 ± 215.4 | 2.3 ± 1.7 | 0.002 |
| Number of mutation[b] | 5,213.8 ± 7,115.7 | 75.7 ± 57.7 | 0.002 |
| POLE or POLD1 exonuclease domain mutant | 46.7% (7/15) | 0% (0/22) | 0.001 |
| MSI | 6/12 (50.0%) | NA | — |
| dMMR | 9/12 (75.0%) | NA | — |

[a]Brinkman index = number of cigarettes smoked/day × number of years smoked.
[b]Mutation number within the coding region of tumor DNA; dMMR: mismatch-repair deficiency.

personal or family history of LS-associated cancers was higher in the hTMB cancer group than in the lTMB cancer group; however, this was not statistically significant (40.0% vs. 18.2%, $P = 0.142$; **Table 4**). TMB levels were higher in the hTMB ECs (157.7) than the hTMB colorectal cancers (67.6); however, this was not a significant difference ($P = 0.924$, by Mann–Whitney *U*).

### *BRAF* mutation, MMR expression, and MSI in LS-associated hTMB cancers

IHC of 4 MMR proteins (MLH1, MSH2, MSH6, and PMS2) and MSI analysis were conducted for 47 hTMB cancers (34 colorectal cancers, 1 small intestine cancer, and 12 ECs) but not performed in 24 hTMB colorectal cancers (*BRAF* V600E mutation positive: 21, uncontacted: 2, and disagreed: 1) and 3 hTMB ECs (disagreed in all 3). Hence, 6 cases of *BRAF* wild hTMB colorectal cancer and 3 cases of hTMB EC were candidates for g.*MMR* target sequencing, but did not undergo the gene examination (Supplementary Tables S1 and S2).

Deficiency in MMR expression was confirmed in 69.7% (23/33) of hTMB colorectal cancers, of which 95.7% (22/23) showed high levels of MSI (**Table 3**), except for one MSH6-repressed colorectal cancer [in germline *MSH6* (g.*MSH6*) p.W413S (c.1238G > C) variant] with low levels of MSI (C-25 in Supplementary Table S1). *MLH1* gene-expression levels were significantly lower in hTMB colorectal cancers with *BRAF* V600E mutations (−2.0 ± 0.8) than those without (−0.1 ± 0.8; $P < 0.0001$; **Table 3**).

Meanwhile, dMMR was recognized in 75% (9/12) of hTMB ECs, of which 66.7% (6/9) were positive for MSI (**Table 4**). All 4 *BRAF* mutations recognized in the 3 hTMB ECs were not registered in ClinVar and HGMD, and unknown for pathogenicity. Discrepancy of MMR IHC and MSI was recognized in 25% (3 of 12) of hTMB ECs; g.*MSH6* variants were confirmed in these 3 EC cases with repressed MSH6 expression but without MSI (E-4, 8, and 9 in Supplementary Table S2). The prevalence of MSI and dMMR was not significantly different between the colorectal cancers and ECs ($P = 0.31$ and $P = 0.73$, respectively; **Tables 3** and **4**).

### Detection of Lynch syndrome

Of the 2,501 patients with surgically resected cancers, the g.*MMR* variant was detected in 16 (0.6%) patients; all 16 patients had LS-related cancers [9 cases of colorectal cancer (1.1%; 9/826), 6 cases of EC (16.2%; 6/37), and one case of small intestine cancer (14.3%; 1/7); **Table 5**]. A g.*MLH1* variant (exon 5 deletion) could not be detected either by universal sequencing or by the TMB-based method, because the variant was a large deletion, undetectable by ion-semiconductor sequencing, and this colon cancer did not have hTMB (TMB = 8.5). However, the detection rate (sensitivity) of the g.*MMR* variant largely differed between universal g.*MMR* sequencing (56.3%, 9/16) and TMB-based target g.*MMR* sequencing (81.3%, 13/16; $P = 0.127$, **Table 5**), as in the following.

#### *Universal g.*MMR *sequencing*

Depth of coverage in current WES for germline DNA was 122.1 ± 860.7 (average ± SD). Universal exome sequencing detected 9 g.*MMR* variants (0.4%) in the 2,501 cancer cases: 6 colorectal cancers and 3 ECs (**Table 5**). These variants were all single-nucleotide substitutions within the exome and a few bases into the intron site. A case of EC with an *MSH6* (IVS6+1G>T) variant (**Table 5**), which was detected via universal *g.MMR* sequencing (ion-proton method), could not be detected via TMB-based g.*MMR* target sequencing, as this EC was negative for MMR protein repression and MSI. Meanwhile, this EC patient previously developed a colorectal cancer showing MSH6 protein deficiency and high-level MSI.

#### *Target sequencing and MLPA of g.*MMR *in cases with hTMB,* BRAF *wild-type, and MMR-deficient cancers*

The target sequencing and MLPA of g.*MMR* genes was performed on d.MMR, TMB-high, and LS-associated cancers as well as with an additional condition of wild-type *BRAF* in cases of colorectal cancer. Depth of coverage for g.*MMR* target sequencing was 7,143.4 ± 2,944.0 (average ± SD). This strategy detected LS in 13 cases (0.5%) in the 2,501 cancer cases: 8 colorectal cancers, 5 ECs, and 1 small intestine cancer (**Table 5**). This method detected a short to large range of deletions and duplications, such as *MLH1* (exon13–19 del), *MSH2* (p.E290Dfs*2), *PMS2* (p.I18Sfs*34), and *MSH2* (exon7 dup), which could not be discovered with ion-semiconductor sequencing. However, this TMB-based approach missed 2 LS patients, as an EC that developed in 1 patient [*MSH6* (IVS6+1G>T)] was not associated with the MMR pathway, and a colorectal cancer developed in another patient showed moderate levels of TMB (8.5; **Table 5**).

Germline *MMR* sequencing was performed for 30 hTMB LS-associated cancers with repressed or lost MMR protein expression (20 colorectal cancers, 1 small intestine cancer, 9 ECs;

**Table 5.** Total g.*MMR* variants detected in 2,501 cancer patients: Comparison of strategies for g.*MMR* variant detection.

| | | | Detection of g.*MMR* variant | | |
|---|---|---|---|---|---|
| | **Case No.** | **g.*MMR* variant** | **Universal sequencing** | **TMB-based-targeted sequencing** | **Others** |
| Colorectal cancer ($n = 826$) | C-51 | *MLH1 (p.E23*)* | Yes | Yes | |
| | C-48 | *MLH1 (IVS6+2T>C)* | Yes | Yes | |
| | C-47 | *MLH1 (p.A681T)* | Yes | Yes | |
| | | *MLH1 (exon5 del)*[a] | *No* | *No* | Yes |
| | C-12 | *MLH1 (exon13–19 del)* | *No* | Yes | |
| | C-29 | *MSH2 (exon7 dup)* | *No* | Yes | |
| | C-9 | *MSH6 (p.E376*)*[b] | Yes | Yes | |
| | C-25 | *MSH6 (p.W413S)*[c] | Yes | *No* | |
| | C-16 | *MSH6 (p.R482*)* | Yes | Yes | |
| | | | Frequency of detected g.*MMR* variants | | |
| | | | 0.7% (6/826) | 0.85% (7/826) | 0.1% (1/826) |
| Endometrial cancer ($n = 37$) | E-14 | *MSH2 (p.K235Ffs*9)*[b] | *No* | Yes | |
| | E-10 | *MSH2 (p.E290Dfs*2)* | *No* | Yes | |
| | E-4 | *MSH6 (p.E376*)*[b] | Yes | Yes | |
| | E-8 | *MSH6 (p.W413S)*[c] | Yes | Yes | |
| | E-9 | *MSH6 (p.F1088Sfs*2)* | *No* | Yes | |
| | | *MSH6 (IVS6+1G>T)*[d] | Yes | *No* | |
| | | | Frequency of detected g.*MMR* variants | | |
| | | | 8.1% (3/37) | 13.5% (5/37) | |
| Small intestine cancer ($n = 7$) | S-1 | *PMS2 (p.I18Sfs*34)* | *No* | Yes | |
| | | | Frequency of detected g.*MMR* variants | | |
| | | | 0% (0/7) | 14.3% (1/7) | N/A |
| Incidence of detected g.*MMR* variant ($n = 2,501$) | | | 0.4% (9/2,501) | 0.5% (13/2,501) | 0.04% (1/2,501) |
| Detection rate of g.*MMR* variant ($n = 16$) | | | 56.3% (9/16)[e] | 81.3% (13/16)[e] | 6.3% (1/16) |

[a]A variant of *MLH1* (exon 5 del) was not detected either by the hTMB approach or by the universal g.*MMR* sequencing approach as the variant was a large deletion and the TMB was 8.5 (<10/Mb). This variant was detected in cases of family history, MSI, and MMR IHC using the MLPA method.
[b]Pathogenicity of the two newly detected variants was evaluated on the basis of ACMG guideline.
[c]Pathogenicity of one probable pathogenic variant was evaluated on the basis of ACMG guideline.
[d]An MSH6 (IVS6+1G>T) variant was detected only by universal g.*MMR* sequencing but not by the TMB-based approach, as the analyzed endometrial cancer was not developed via the MMR pathway. However, her previous colorectal cancer was positive for MSH6 deficiency and MSI.
[e]$P = 0.13$.

Supplementary Tables S1 and S2). Six cases of *BRAF* V600E-mutant hTMB colorectal cancer were exceptionally analyzed for g.*MMR* sequencing, and one pathogenic variant (*MSH6* p.W413S, C-25 in Supplementary Table S1) was detected. Within the remaining 24 hTMB cases sequenced for g.*MMR*, the pathogenic variant was detected in 13 cases (54.2%; 7 colorectal cancers, 1 small intestine cancer, and 5 ECs; Supplementary Tables S1 and S2). The results of MMR IHC and g.*MMR* sequencing were largely concordant, except for one case (C-44) that had a benign *MLH1* variant (p.Q701K; Supplementary Table S1); otherwise, MLH1 lost cancers that can be affected by *MLH1* methylation.

In 14 MMR-deficient hTMB cancers in the LS patients (Supplementary Tables S1 and S2), somatic deleterious *MMR* gene mutations were detected in 9 cancers (64.3%: 6 of 8 colorectal cancers, 1 of 1 small intestine cancer, and 2 of 5 of ECs), by either of point mutation, deletion, frameshift, or LOH.

### *Pathogenic evaluation of novel g.*MMR *variants*

In this study, 2 novel germline variants [*MSH2* (p.K235Ffs*9) and *MSH6* (p.E376*); **Table 5**] were deemed to be pathogenic (PVS1) based on the ACMG guidelines (32), as they were thought to create nonsense mutations. One probable pathogenic variant (*MSH6* p.W413S), which was seen in one colorectal cancer case (C-25 in Supplementary Table S1) and one EC case (E-8 in Supplementary Table S2), was deemed to be likely pathogenic, as per the ACMG guidelines, and its repressed MSH6 expression.

## Discussion

Today, TMB has been garnering increasing attention in cancer precision medicine due to its critical role as a key biomarker in predicting the response to immune-checkpoint inhibitors (1–3). Cristescu and colleagues (34) defined hTMB as >102.5 mutations/exome for predicting good responders to immune-checkpoint inhibitors; however, the Cancer Genome Atlas (10, 30) defined hTMB as ≥10 mutations/Mb for detecting MSI-high and *POL*-mutant cancers (LS detection rate: 81.3%). If we set hTMB as ≥20 mutations/Mb, ≥50 mutations/Mb, or ≥100 mutations/Mb, detection rate of LS was 75.0% (12/16), 25.0% (4/16), or 6.3% (1/16), respectively. Hence, excessively high cutoff level of TMB will miss LS patients. Actually, most of our hTMB cancer groups (TMB ≥10) exhibited hypermutators and ultramutators and shared several demographic features, such as frequent LS-associated cancers and skin cancer (**Table 2**), modestly increasing cases meeting the revised Bethesda criteria (**Table 3**), and cases with personal and family histories of LS-related cancers (**Table 4**). Although TMB is affected by mutagenic exposures, such as smoking for lung cancer (35, 36) and ultraviolet light for skin cancer (5, 35), current results demonstrated no correlation between Brinkman index and

TMB in cases with LS-related cancer (**Tables 3** and **4**). Aging also increases the level of TMB mildly (37), but it rather showed negative correlation in current cases of endometrial cancer (**Table 4**), probably due to the high proportion of *POL*-mutant cancers (38). To comprehend these findings, the hTMB LS-associated cancer patients were thought to be good candidates for LS screening, and we detected LS in 54.2% of the patients tested with target g.*MMR* sequencing and MLPA. The sensitivity of this strategy (81.3%) was superior to universal g.*MMR* sequencing using the ion-semiconductor method (56.3%), although this difference did not reach statistical significance (**Table 5**).

Formerly, the diagnosis of LS had been made based on the selection of high-risk individuals based on the patients' demographics and tumor histologic findings, according to the Amsterdam II criteria or revised Bethesda guidelines (14). However, due to its low detection rate, the Evaluation of Genomic Application in Practice and Prevention (EGAPP) working group does not currently recommend using this strategy for LS identification (39). In the past decade, universal screening has been attempted for LS-related cancer patients or those with age-specific conditions, specifically using MSI analysis and/or MMR IHC in their tumors. MMR IHC seems to have some advantage over MSI, as cancers with *MSH6* or *PMS2* mutations have shown repressed protein expression but sometimes lack MSI (refs. 40, 41; Supplementary Table S2: E-4, 8, and 9 in the current study). However, such universal screening is not fully diffused, even in the USA, and is limited to several cancer centers and some departments at core hospitals (42). In familial cancer clinics, blood relatives are simply diagnosed by examining known g.*MMR* variants. However, new LS probands need to be diagnosed based on deleterious g.*MMR* variants, with prior screening of an MMR deficiency or microsatellite instability in their cancers, besides with an additional condition of *MLH1* promoter methylation or *BRAF* mutation in cases of colorectal cancers (43, 44).

Major cancer centers in the USA have demonstrated pathogenic g.*MMR* variants in 0.5% (45) to 0.7% (6) of advanced cancer patients who underwent clinical sequencing for precision medicine. The universal g.*MMR* sequencing seemed to be most sensitive as it directly determines g.*MMR* genes; however, several issues appeared in this study. First, short-read NGS analysis poorly detects large indels, especially when using ion-semiconductor sequencing (46). As described in **Table 5**, ion-semiconductor sequencing for g.*MMR* genes detected only single-nucleotide variants but missed large deletions and duplications (6 of 16 variants, 37.5%) that were detectable via target sequencing using the MLPA method. To date, WES has dramatically improved the diagnostic yield; however, WES cannot fully delineate certain DNA changes (e.g., structural rearrangement, copy-number variants, tandem-repeat expansions, and variants within the intronic and regulatory regions; refs. 47, 48). Whole-genome sequencing or paired genomic and transcriptomic NGS can solve these issues (48). Recent advance in long-read sequencing technologies, such as single-molecule real-time sequencing developed by Pacific Biosciences and nanopore sequencing by Oxford Nanopore Technologies, enable to detect large genetic alterations (1 bp to 1 million bp) and cover the role of MLPA in addition to NGS, but with high cost and high error rate (5%–15%; ref. 49). Hence, within the context of precision medicine, g.*MMR* sequencing with NGS alone is not optimal for LS detection and should be coupled with TMB. In the place of TMB, MSI can be another option. However, as previously mentioned, universal MSI analysis misses g.*MSH6 and* g.*PMS2* variants that are often seen in ECs (50) and could be detected using the hTMB-based approach (Supplementary Table S2). The setting of hTMB can be adjusted, as current condition (hTMB: ≥10) missed one g.*MLH1* variant with moderate levels of TMB (8.5; **Table 5**). The *BRAF* wild-type condition in colorectal cancer cases is effective and recommended in the guidelines for detecting LS (13, 44); however, a *BRAF* mutation does not always indicate the existence of *MLH1* methylation (4) and can also occur in LS cancer patients (C-25 in Supplementary Table S1). Even with *MLH1* hypermethylation, LS cannot be completely denied (51). The last pitfall is that not all tumors in LS patients develop through a mismatch-repair pathway, as shown by an EC case with a g.*MSH6* IVS6+1G>T variant (**Table 5**), which could only be detected via universal g.*MMR* sequencing and not by either MSI, TMB, or MMR IHC. When predicted findings are obtained in cases with multiple LS-associated cancers, additional MMR IHC should be considered.

In precision medicine, germline DNA sequencing is not always obtained upon the first step, but tumor DNA sequencing is usually done. Hence, the prediction of germline-derived mutation has been pursued in somatic DNA information for detecting hidden inherited cancers (28). This process is routinely done in precision medicine prior to genetic counseling and further attempts at family surveillance. Mandelker and colleagues (28) compared the mutations of somatic and germline DNAs from inherited cancer-associated genes in 17,152 unselected cancer cases (MSK-IMPACT), predicting germline-derived somatic mutations with high sensitivity. They refined their candidates for the germline test into a group with 62.7% sensitivity (653 true germline variants out of 1,042 candidate cancer cases) based on variant allele frequency, minor allele frequency, patient's age, and cancer type. However, by current ion-semiconductor sequencing, only 64.3% (9/14) of LS patients demonstrated somatic mutations in the corresponding *MMR* genes (Supplementary Tables S1 and S2), indicating not only a lower positive predictive value but a low sensitivity to detect LS patients. Undetected somatic *MMR* alteration can be explained by methylation of *MLH1* (52), *MSH2* (53), and/or deletion of *EPCAM* (54). We also need to bear in mind that some of structural genomic rearrangement cannot be detected by NGS analysis (55). In this sense, TMB-based target g.*MMR* sequencing is thought to be an efficient strategy for detecting LS in the setting of precision medicine. When cancer DNA demonstrates high levels of TMB, MMR IHC should be applied after checking for *BRAF* wild status, followed by a germline examination for the corresponding *MMR* gene, conducted through genetic counseling.

The current study included several limitations. The authors presented all registered cases during the study period (2014–2017), but the studied cancer patients could be divided by their entry conditions: patients with surgically resectable cancer and those with tumors large enough to receive $5 \times 5 \times 5$ mm$^3$ study material without disturbing the pathologic diagnosis. Therefore, the number of cases entered by cancer type was not consistent among various cancer types (**Table 1**). TMB-high cancers are biologically less aggressive (10, 56), current cases were diagnosed within the operable stage and therefore may have higher incidence of high MSI and hTMB than entire cases of colorectal and ECs. Universal g.*MMR* sequencing was done using the ion-semiconductor sequencing method, which is useful for single-nucleotide substitutes and short indels, but may have a drawback in its detection of a large range of nucleotide alterations (46). The evaluation of pathogenicity for each germline variant was conducted based on the HGMD and ClinVar databases during the study period and is changeable in the future.

In conclusion, the current study demonstrated the critical role of TMB in detecting LS by its increasing sensitivity to simple universal NGS sequencing within the setting of cancer precision medicine. This strategy can also detect PPAP (11, 57) and constitutional

mismatch-repair deficiency syndrome (CMMRD; ref. 58) effectively. By further applying somatic MLPA for *MMR* genes, patients with Lynch-like syndrome (59, 60) can be confirmed. TMB-based cancer sequencing is also recommended due to its treatment effects, such as predicting the effect of immune-checkpoint inhibitors (3, 61).

## Authors' Disclosures

No disclosures were reported.

## Authors' Contributions

**Y. Kiyozumi:** Conceptualization, data curation, methodology, and writing–original draft. **H. Matsubayashi:** Conceptualization, data curation, formal analysis, methodology, writing–original draft, writing–review and editing. **S. Higashigawa:** Formal analysis. **Y. Horiuchi:** Formal analysis. **N. Kado:** Resources. **Y. Hirashima:** Resources. **A. Shiomi:** Resources. **T. Oishi:** Resources and formal analysis. **S. Ohnami:** Formal analysis and investigation. **K. Ohshima:** Formal analysis and investigation. **K. Urakami:** Formal analysis and investigation. **T. Nagashima:** Data curation and formal analysis. **K. Yamaguchi:** Supervision and project administration.

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