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A subset of patients with MSS/MSI-low-colorectal cancer showed increased CD8(+) TILs together with up-regulated IFN-γ

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Abstract. A small subset of patients with proficient mismatch repair (pMMR)/microsatellite stable (MSS)-colorectal cancer (CRC) benefit from immunotherapy with anti-programmed cell death 1 (PD-1)/programmed death ligand 1 (PD-L1) blockade. Therefore, the aim of the current study was to evaluate the immune status of patients with pMMR/microsatellite instability-low (MSI-L)/MSS-CRC and deficient MMR (dMMR)/MSI-high (MSI-H)-CRC in order to identify responders to anti-PD-1/PD-L1 inhibitors. The current study used a dataset downloaded from The Cancer Genome Atlas (TCGA) as well as 219 clinical tissue samples to investigate the infiltrating grade of cluster of differentiation (CD) 4 and CD8 tumor infiltrating lymphocytes (TILs), the expression levels of PD-L1 and PD-L2, the interferon-γ (IFN-γ) and CD8 T effector gene signatures, and the phosphorylated signal transducer and activator of transcription 1 (p-STAT1) status in patients with pMMR/MSI-L/MSS-CRC and dMMR/MSI-H-CRC. Analysis of TCGA dataset revealed that the mRNA expression levels of PD-L1 and PD-L2, the IFN-γ gene signature and the CD8 T effector gene signature were significantly upregulated in MSI-H tumors compared with MSI-L/MSS tumors. Additionally, a subpopulation of patients with upregulation of the IFN-γ and CD8 T effector gene signatures was observed in those with MSI-L/MSS-CRC. Immunohistochemical staining of the clinical samples revealed a subpopulation of patients with pMMR-CRC that were positive for PD-L1 and p-STAT1, and whom had levels of elevated CD8(+) and CD4(+) TILs infiltration similar to those observed in patients with dMMR-CRC. The results obtained in the current study suggested that a subpopulation of patients with MSI-L/MSS-CRC and pMMR-CRC with upregulated IFN-γ and CD8 T effector gene signatures may benefit from immunotherapy with antibodies against PD-1 and PD-L1.

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Abbreviations: CRC, colorectal cancer; dMMR, deficient mismatch repair; ICIs, immune checkpoint inhibitors; IHC, Immunohistochemistry; IFN-γ, interferon-γ; JAK, Janus-activated kinase; MSI-H, microsatellite instability-high, MSI-L, MSI-low; MSS, microsatellite stable; mAbs, monoclonal antibodies; p-STAT1, phosphorylated signal transducer and activator of transcription 1; pMMR, proficient MMR; PD-1, programmed cell death 1; PD-L1, programmed death ligand 1; Treg, regulatory T; TCGA, The Cancer Genome Atlas; TILs, tumor infiltrating lymphocytes; TMB, tumor mutation burden

Key words: colorectal cancer, interferon-γ, microsatellite instability, microsatellite stable, programmed cell death 1, proficient mismatch repair, tumor infiltrating lymphocytes

Introduction

Colorectal cancer (CRC) was the third most common type of cancer and the fourth leading cause of cancer-associated mortality worldwide in 2012 (1,2). CRC is classified into two categories: i) The microsatellite instability-high (MSI-H) group, which is caused by defects in the DNA mismatch repair (MMR) system and accounts for ~15% of tumors; and ii) the microsatellite stable (MSS) group, which exhibits chromosomal instability and accounts for the remaining 85% of tumors (3-5). At the protein level, MSI-H is similar to deficient MMR (dMMR) status, while MSI-low (MSI-L)/MSS are similar to proficient MMR (pMMR) status (6,7).

Immunotherapy with anti-programmed cell death 1 (PD-1)/programmed death ligand 1 (PD-L1) monoclonal antibodies (mAbs) is a standard therapeutic strategy in various types of cancer, including lung cancer, gastric cancer and renal cell carcinoma (8-10). Although the results of initial trials of immune checkpoint inhibitors (ICIs) in CRC were not particularly promising (11,12), dMMR/MSI-H patients with CRC are currently considered to be candidates for anti-PD-1/PD-L1 immunotherapy (13,14). In a phase II clinical trial, treatment with the anti-PD-1 mAb pembrolizumab resulted in a

significant clinical response in patients with dMMR-CRC, but not those with pMMR-CRC (13). Similar results were observed in dMMR/MSI-H tumors across 12 different cancer types (15). Furthermore, a recent phase II trial demonstrated the clinical benefit of the PD-1 mAb nivolumab in patients with dMMR/MSI-H-CRC (14). Therefore, immunotherapy with ICIs targeting the PD-1/PD-L1 axis may be a promising treatment option for patients with dMMR/MSI-H solid tumors of varying histological origins.

In general, dMMR/MSI-H tumors in CRC are thought to respond to ICIs due to their highly immunogenic nature (14). For example, dMMR/MSI-H results in the accumulation of several mutations in microsatellites spread along the genome, and produces neo-antigens that produce a highly antigenic microenvironment with a high density of tumor infiltrating lymphocytes (TILs) (3,5,16,17). Moreover, simultaneous expression of multiple immune checkpoint molecules, including PD-1 and PD-L1, has been reported in dMMR/MSI-H-CRC (3,5,16,17). Consequently, in patients with dMMR/MSI-H-CRC, treatment with ICIs targeting the PD-1/PD-L1 axis reactivates anti-tumor specific T cells that subsequently attack tumor cells.

Previous studies revealed that ICIs targeting the PD-1/PD-L1 axis were ineffective at treating MSS/pMMR-CRC (11,12). Clinical studies have established MSI status as a putative response biomarker for PD-1 blockade, with progression free survival rates of up to 78% reported in patients with MSI-H-CRC, compared with 11% of patients with MSS-CRC (13,17). However, other studies have demonstrated that ~10% of patients with MSS/pMMR-CRC exhibit a response to PD-1/PD-L1 inhibitors (13,17). Based on the aforementioned clinical evidence, the aim of the current study was to compare the immune statuses of patients with MSI-L/MSS or pMMR-CRC with MSI-H or dMMR-CRC, in order to identify responders with MSI-L/MSS or pMMR-CRC as distinct biomarker-defined populations. The current study therefore investigated the infiltrating grade of CD4 and CD8 TILs, the expression levels of PD-L1 and PD-L2, the interferon-γ (IFN-γ) and CD8 T effector gene signatures, and the phosphorylated signal transducer and activator of transcription 1 (p-STAT1) status in patients with MSI-L/MSS or pMMR- CRC compared with those with MSI-H or dMMR-CRC.

Materials and methods

The Cancer Genome Atlas (TCGA) dataset analysis. Level 3 Illumina RNA-Seq data (RNA-Seq V2 RSEM normalized) for colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) in TCGA were downloaded through the cBioPortal (www.cbioportal.org) in July 2018 (18) and consisted of 342 samples. MSI testing data (MSI-H, MSI-L or MSS) for each TCGA tumor were obtained from a previous study by Liu *et al* (19). The current study analyzed multi-gene expression signatures, including the CD8 T effector gene signature [CD8A molecule (*CD8A*), CD8B molecule (*CD8B*), eomesodermin (*EOMES)*, granzyme A (*GZMA)*, granzyme B *(GZMB)*, interferon γ (*IFNG*) and perforin 1 (*PRF1)*] and the IFN-γ gene signature [indoleamine 2,3-dioxygenase 1 (*IDO1*), C-X-C motif chemokine ligand (*CXCL*) 9 and 10, major histocompatibility complex class II DR α (*HLA-DRA*), *STAT1* and *IFNG*] (20,21). The IFN-γ gene signature was identified from three clinical studies investigating pembrolizumab, and focused on genes associated with antigen presentation and cytotoxic activity in 220 patients with nine types of cancer (21). The CD8 T effector gene signature was previously established to identify activated T cells such as cytotoxic T lymphocytes (20). The signature scores were calculated by averaging the expression levels of the genes included in each signature (22), excluding *HLA-DRA* expression values, as these were not available in TCGA RNA-seq data.

Patient samples. Formalin-fixed paraffin-embedded tissue samples from 219 patients with primary CRC, who had undergone surgical resection without preoperative chemotherapy or radiotherapy in Fukushima Medical University Hospital (Fukushima, Japan) between January 2007 and December 2013 were analyzed in the current study. A total of 138 men and 81 women (mean age, 67.8±12.4 years; age range, 27-94 years), were included (Table I). Patients with stage 0 CRC were excluded. Clinical and pathological data were retrospectively obtained from medical records, with the last follow-up in April 2017 (Table I).

Immunohistochemistry (IHC). Tissue blocks were cut into 4-μm-thick sections and were subsequently deparaffinized by three washes of 3 min with xylene at room temperature and rehydrated by decreasing gradient of ethanol at room temperature. Sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity. For CD4, CD8 and forkhead box P3 (FOXP3) staining, antigen retrieval was performed by autoclaving the sections for 5 min at 105˚C in target retrieval solution (pH 9.0; Dako; Agilent Technologies, Inc.). For PD-L1 staining, antigen retrieval was performed by autoclaving the sections for 10 min at 120˚C in target retrieval solution (pH 9.0). For p-STAT1 staining, antigen retrieval was performed by autoclaving the sections for 5 min at 105˚C in citrate buffer solution (pH 6.0). Tissue sections were subsequently incubated with the following primary antibodies directed against CD4 (clone 4B12; cat. no. M7310; 1:100; Dako; Agilent Technologies, Inc.), CD8 (clone C8/144B; cat. no. M7103; 1:100; Dako; Agilent Technologies, Inc.), FOXP3 (clone 236A/E7; cat. no. ab20034; 1:200; Abcam), PD-L1 (clone E1L3N; cat. no. 13684S; 1:400; Cell Signaling Technology, Inc.) and p-STAT1 (clone D3B7; cat. no. 8826S; 1:800; Cell Signaling Technology, Inc.) at 4˚C overnight. Following the primary antibody incubation, the sections were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (Envision + System-HRP; cat. no. K4003 or K4001; ready to use; Dako; Agilent Technologies, Inc.) for 30 min at room temperature. The sections were then stained with diaminobenzidine (Dojindo Molecular Technologies, Inc,) at room temperature for 10 min and subsequently counterstained with Mayer's hematoxylin solution (cat. no. 131-09665; Wako Pure Chemical Industries, Ltd.) at room temperature for 1 min. Sections in which the primary antibodies were replaced with PBS were used as negative controls. Esophagus and stomach cancer tissue sections served as positive controls (23).

Table I. Clinical features of the patients (n=219).

Clinical features	Number of patients
Sex	
Male	138
Female	81
TNM stage[a]	
I	49
II	72
III	74
IV	24
Degree of differentiation	
Moderate/high differentiation	204
Poor differentiation/undifferentiated	15
Lymphatic metastasis[a]	
N0	126
N1-3	93
Degree of invasion[a]	
T1	25
T2	39
T3	95
T4	60

TNM, tumor-node-metastasis. [a]The Japanese Classification of Colon and Rectum Carcinoma was defined according to the Japanese Society of the Colon and Rectum (The 9th Edition).

Assessment of IHC staining. IHC analysis was performed by four independent observers (TK, KS, LY and EE) who were blinded to the clinical data. The TILs at the invasive front region of the tumor were reviewed in four independent areas using light microscope at a magnification of x400. PD-L1 expression was evaluated by assessing membranous staining without cytoplasmic staining and tumor specimens were considered to be PD-L1-positive when >1% of the tumor cells exhibited membranous staining of any intensity (24). p-STAT1 staining was evaluated by assessing nuclear staining of the tumor cells. Positive staining for p-STAT-1 was defined by the presence of positive nuclear staining in tumor cells. CD4, CD8 and FOXP3 expression was evaluated by counting the number of stained lymphocytes from four independent areas.

Determination of MMR status. IHC for MMR proteins, including mutL homolog 1, mutS homolog 2 and 6, and PMS1 homolog 2 mismatch repair system component, was performed as previously described (25). Loss of a MMR protein was defined as the absence of nuclear staining of tumor cells in the presence of positive nuclear staining of normal colon mucosa cells or stromal cells. Tumors demonstrating the loss of at least one MMR protein were designated as dMMR, and tumors with intact MMR protein expression as pMMR.

Statistical analysis. An unpaired Student's t-test was used to compare two groups and one-way analysis of variance followed by a Tukey's post hoc test was used to compare multiple groups. The data are presented as the means ± standard deviation. The correlation of IFN-γ or CD8 T effector gene signatures with the expression of PD-L1 and PD-L2, STAT1 and Janus-activated kinase (JAK) 1 or 2, and the correlation of the IFN-γ gene signature with the CD8 T effector gene signature were assessed using scatter diagrams and Pearson's correlation test. Associations between p-STAT1 and PD-L1 expression were assessed using the χ^2 test. All statistical analyses were conducted using GraphPad Prism software (version 7.0; GraphPad Software, Inc.). P-values were two-sided and $P<0.05$ was considered to indicate a statistically significant difference.

Results

A subset of patients with MSI-L/MSS-CRC exhibit IFN-γ and CD8 T effector gene signature upregulation. TCGA COADREAD dataset consisted of 342 samples, including 51 MSI-H (14.9%) and 291 MSI-L/MSS (85.1%) tumor samples. The mRNA expression levels of PD-L1 and PD-L2 were significantly upregulated in MSI-H tumors compared with MSI-L/MSS tumors (Fig. 1A). The IFN-γ gene signature consisting of *IDO1, CXCL10, CXCL9, STAT1* and *IFNG*, and the CD8 T effector gene signature consisting of *CD8A, CD8B, EOMES, GZMA, GZMB, IFNG* and *PRF1* were subsequently assessed. The two gene signatures were more prevalent in MSI-H tumors compared with MSI-L/MSS tumors (Fig. 1A). There was a significant variation in the IFN-γ gene signature in the MSS/MSI-L group. A subpopulation with a high IFN-γ gene signature in the MSS/MSI-L group, with expression levels of the IFN-γ gene signature similar to those in the MSI-H group was identified (Fig. 1B). Similarly, there was a subset of patients with a high CD8 T effector gene signature in the MSS/MSI-L group, with expression levels similar to those in the MSI-H group (Fig. 1B). The results obtained in the current study suggest that there is a subpopulation of patients with upregulated CD8 T effector and IFN-γ gene signatures in MSI-L/MSS-CRC.

Correlations of PD-L1 and PD-L2 with the IFN-γ and CD8 T effector gene signatures. There were significant positive correlations between the IFN-γ gene signature and PD-L1, between the IFN-γ gene signature and PD-L2, between the CD8 T effector gene signature and PD-L1, between the CD8 T effector gene signature and PD-L2, and finally between the CD8 T effector and IFN-γ gene signatures in all 342 CRC cases (Fig. 2A). The current study demonstrated that a subset of patients with CD8 T effector and IFN-γ gene signature upregulation in the MSI-L/MSS-CRC group exhibited upregulation of immune checkpoint molecules, including PD-L1 and PD-L2.

In the present study, there were significant positive correlations between the IFN-γ or CD8 T effector gene signatures and STAT1 or JAK1/2 (Fig. 2B). Furthermore, STAT1 and JAK2 mRNA expression levels were significantly upregulated in MSI-H tumors compared with MSI-L/MSS tumors (Fig. 2C). These findings suggested that the activation of IFN-γ signaling is associated with IFN-γ production and CD8 T cell infiltration within the tumor microenvironment, and that IFN-γ signaling is significantly activated in MSI-H tumors compared with MSI-L/MSS tumors. Since the STAT1 mRNA expression level

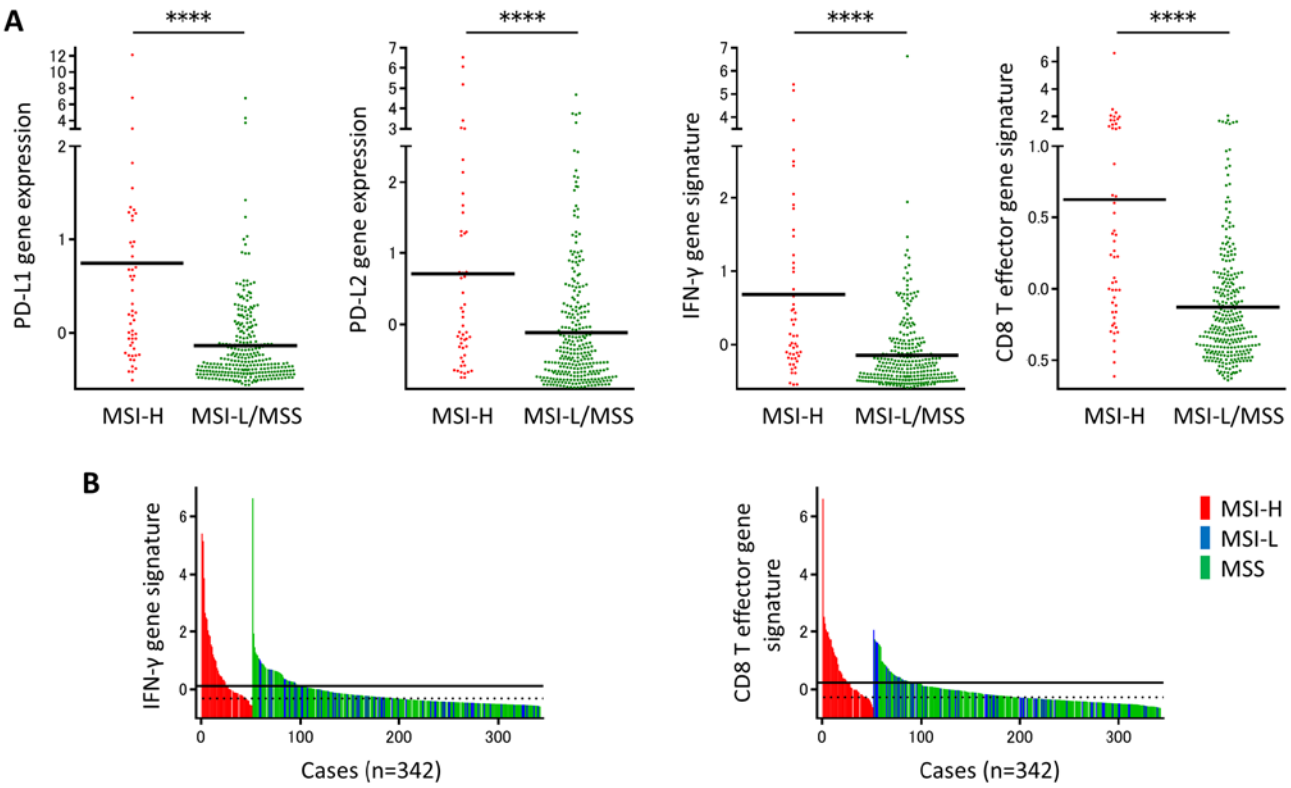

Figure 1. A subset of patients with MSI-L/MSS-CRC revealed upregulation of IFN-γ and CD8 T effector gene signatures. (A) PD-L1 and PD-L2 gene expression levels, and IFN-γ and CD8 T effector gene signatures in The Cancer Genome Atlas colorectal adenocarcinoma tumors, according to MSI status (51 MSI-H and 291 MSI-L/MSS tumors). (B) Subpopulations in MSI-L-/MSS-CRC showed upregulation of IFN-γ and CD8 T effector gene signatures. IFN-γ and CD8 T effector gene signatures were shown in 342 colorectal tumors in relation to MSI status. Individual samples are represented as color bars (red, blue or green) to denote MSI-H, MSI-L or MSS tumors, respectively. Black lines represent the median of each signature in MSI-H tumors, and dotted lines represent the median of each signature in MSI-L/MSS tumors. ****P<0.0001. MSI, microsatellite instability; MSI-L, microsatellite instability-low; MSS, microsatellite stable; CRC, colorectal cancer; IFN-γ, interferon γ; CD, cluster of differentiation; PD-L, programmed cell death ligand; MSI-H, microsatellite instability-high.

was correlated to the highest degree with the IFN-γ and CD8 T effector gene signatures (Fig. 2B), and the binding of IFN-γ to its cognate receptor leads to the phosphorylation-dependent activation of STAT1 (26), the p-STAT1 expression level in clinical samples was analyzed to investigate the effect of IFN-γ in the tumor microenvironment.

A subset of patients in the pMMR-CRC group exhibit increased CD8 and CD4 infiltration. In the current study, a total of 219 patients received colorectal surgery (Table I). IHC was used to classify patients based on MMR protein expression, and a total of 18 (8.2%) and 201 (91.8%) patients were identified to have dMMR- and pMMR-CRC, respectively. Representative IHC staining for MMR proteins are presented in Fig. 3A. Compared with TCGA COADREAD database, the prevalence of dMMR-CRC in the current study was low (14.9 vs. 8.2%). The immune status, including PD-L1 and p-STAT1 in the tumors, and CD4(+), CD8(+) and FOXP3(+) TILs in the tumor microenvironments, was evaluated by IHC as presented in Fig. 3B. There were significant associations between PD-L1 expression and the grade of CD4(+) or CD8(+) TIL infiltration; however, there was no significant association between PD-L1 expression and FOXP3(+) TIL infiltration (Fig. 4A-C). Furthermore, there was a tendency towards a positive association between PD-L1 and p-STAT1 expression levels (Fig. 4D). The immune parameters of patients with dMMR- and pMMR-CRC were compared, a subpopulation of PD-L1(+) and p-STAT1(+) patients with pMMR-CRC (n=26) showed increasing grades of infiltrating CD4(+) or CD8(+) TILs compared with pMMR/others including pMMR-CRC without a subpopulation of PD-L1(+) and p-STAT1(+) patients, which were similar to the levels seen in patients with dMMR-CRC (Fig. 4E and F). Although the number of FOXP3(+) TILs was increased in patients with pMMR-CRC with PD-L1(+) and p-STAT1(+) compared with patients with dMMR-CRC, there were no significant difference between pMMR-CRC/others and dMMR- or pMMR-CRC with PD-L1(+) and p-STAT1(+) regarding FOXP3 TIL infiltration (Fig. 4G). The aforementioned observations in the clinical cohort suggested that there was a subset of patients with pMMR-CRC with elevated CD8(+) and CD4(+) TIL infiltration.

Discussion

There are several potential biomarkers to predict response to ICIs, including tumor mutation burden (TMB), MSI status, PD-L1 expression, CD8 infiltration and the IFN-γ gene signature (27-33). Several clinical studies have established MSI status as a putative response biomarker for PD-1 blockade, and immunotherapy with ICIs against the PD-1/PD-L1 axis is clinically approved for dMMR/MSI-H solid tumors of varying histological origin (13,14,17). dMMR/MSI-H tumors are thought to respond to ICIs for a number of reasons. dMMR/MSI-H tumors have an antigenic microenvironment

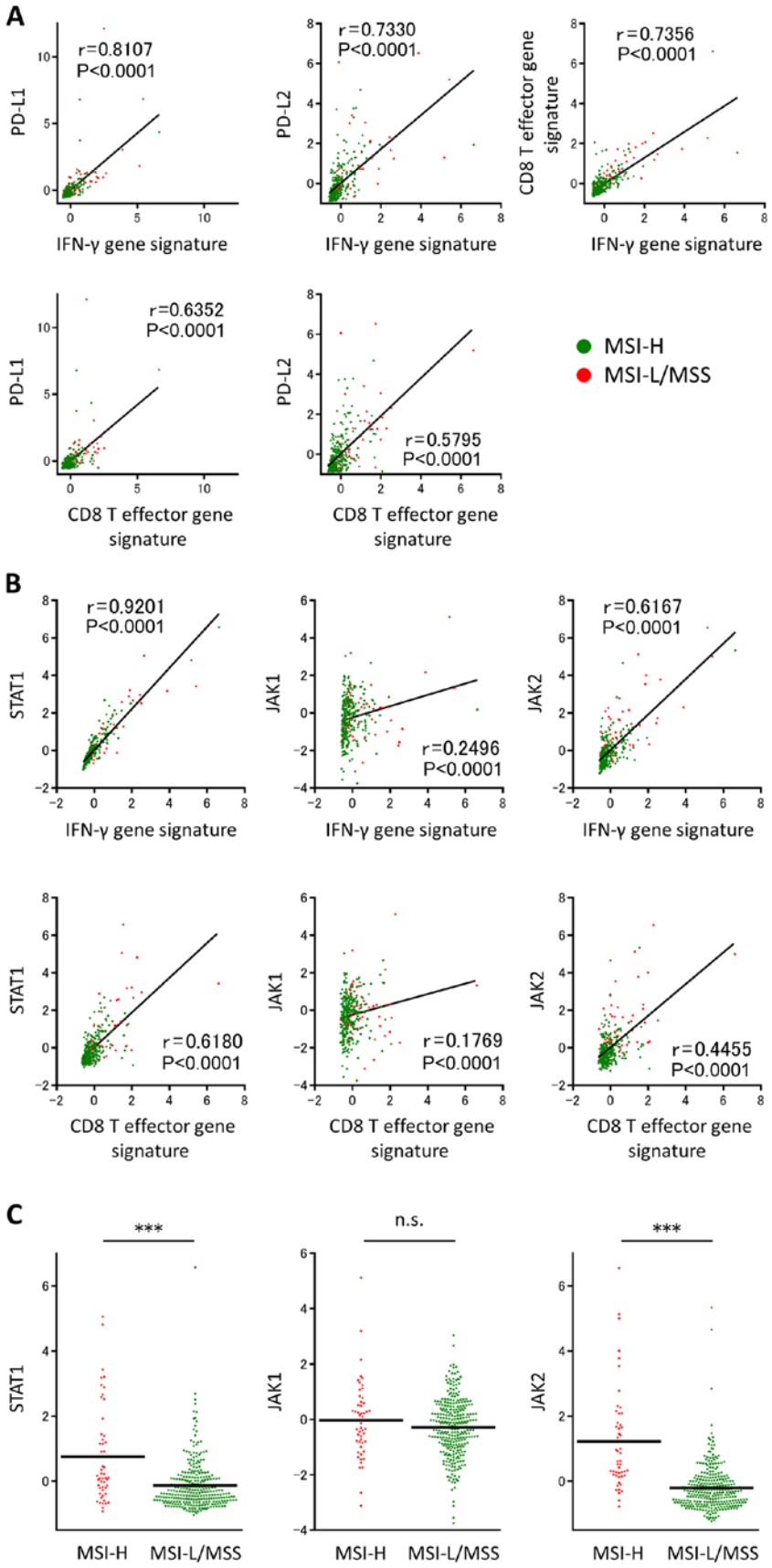

Figure 2. Association of IFN-γ and CD8 T effector gene signatures with the expression of PD-L1 or PD-L2 in TCGA dataset. Individual samples are represented as red or green dots, indicating MSI-H or MSI-L/MSS, respectively. (A) Significant positive correlations were revealed between the IFN-γ or CD8 T effector gene signatures and PD-L1 or PD-L2, and between the IFN-γ and CD8 T effector gene signatures. (B) There were also significant positive correlations between the IFN-γ or CD8 T effector gene signatures and STAT1, JAK1 or JAK2. (C) STAT1, JAK1 and JAK2 gene expression levels in TCGA colorectal adenocarcinoma tumors, according to MSI status. ***P<0.001. CD, cluster of differentiation; IFN-γ, interferon γ; PD-L, programmed cell death ligand; TCGA, The Cancer Genome Atlas; STAT1, signal transducer and activator of transcription 1; JAK, Janus-activated kinase; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, microsatellite stable.

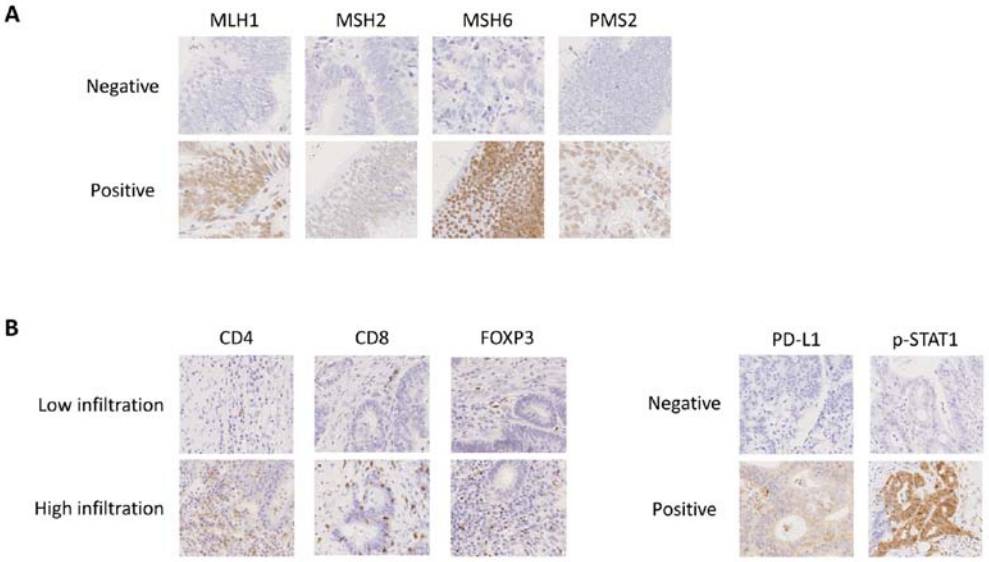

Figure 3. Representative IHC staining. (A) Representative IHC staining of MMR proteins including MLH1, MSH2, MSH6 and PMS2. (B) Representative IHC staining of CD4, CD8, FOXP3, PD-L1, and p-STAT1. Magnification, x400. IHC, immunohistochemistry; MMR, mismatch repair; CD, cluster of differentiation; MLH, mutL homolog; MSH6, mutS homolog 6; PMS2, PMS1 homolog 2 mismatch repair system component; FOXP3, forkhead box P3; PD-L1, programmed cell death ligand 1; p-STAT1; phosphorylated-signal transducer and activator of transcription 1.

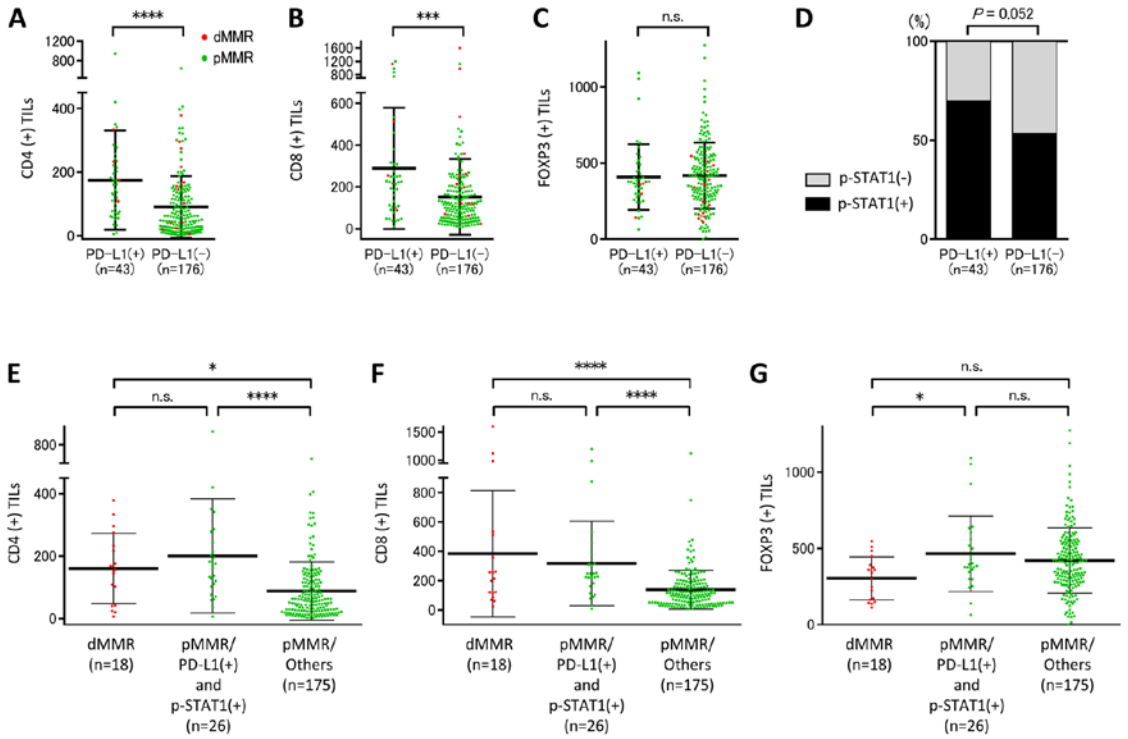

Figure 4. A subset of patients with PD-L1(+) and p-STAT1(+) pMMR-CRC exhibited increased CD4(+) and CD8(+) TIL infiltration as seen in dMMR-CRC. In all patients with CRC, there were significant positive correlations (A) between the number of CD4(+) TILs and PD-L1 expression and (B) between the number of CD8(+) TILs and PD-L1 expression. (C) There was no significant difference between the number of FOXP3(+) TILs and PD-L1 expression and (D) between p-STAT1 and PD-L1 expression on tumor cells. A subpopulation of patients with pMMR-CRC with PD-L1(+) and p-STAT1(+) exhibited increased (E) CD4(+) and (F) CD8(+) TILs, which were similar to levels observed in patients with dMMR-CRC, compared with patients with pMMR-CRC/others. (G) The number of FOXP3(+) TILs was increased in a subpopulation of patients with pMMR-CRC with PD-L1(+) and p-STAT1(+) compared with patients with dMMR-CRC. Error bars represent the means ± SD. *P<0.05, ***P<0.001, ****P<0.0001. PD-L1, programmed cell death ligand 1; p-STAT1, phosphorylated-signal transducer and activator of transcription 1; MMR, mismatch repair; pMMR, proficient MMR; CRC, colorectal cancer; CD, cluster of differentiation; TIL, tumor infiltrating lymphocyte; dMMR, deficient MMR; FOXP3, forkhead box P3; n.s., not significant.

with a high density of TILs and express multiple immune checkpoint molecules, including PD-1 and PD-L1, due to mutation-induced neo-antigens (3,5,16,17). The present study used clinical samples and TCGA database to reveal that dMMR/MSI-H tumors have immunogenic tumor microenvironments and exhibit upregulated PD-L1 and PD-L2

expression levels, and an increase in p-STAT1, and increased infiltration of CD8 T cells compared with pMMR/MSS tumors, and that these factors are closely associated with IFN-γ production within the tumor microenvironment. Since the binding of IFN-γ to its cognate receptor leads to the phosphorylation-dependent activation of JAK1/2 and STAT1, and that p-STAT1 forms subsequently homodimers that translocate to the nucleus and activate the transcription of IFN-γ-stimulated genes (26), p-STAT1 expression was investigated in the present study. These observations are in line with previous studies investigating gastric cancer, which reported that the upregulation of PD-L1 and PD-L2 is significantly associated with IFN-γ production and CD8 T cell infiltration within the tumor microenvironment (34,35).

However, previous studies revealed that in pMMR/MSS-CRC, a small subset of patients may still benefit from immunotherapy with anti-PD-1/PD-L1 antibodies (13-15). Therefore, the identification of distinct biomarker-defined populations with pMMR/MSS-CRC may improve patient outcome. The present study revealed a subset of patients with MSI-L/MSS-CRC with upregulated CD8 T effector and IFN-γ gene signatures, as seen in MSI-H-CRC. Previous studies reported that the number of $CD8^+$ TILs was increased in MSI-CRC compared with MSS-CRC (36-38). Also, De Smedt *et al* (36) used IHC to demonstrate that there were no significant differences in the number of CD4(+) TILs in MSI- or MSS-CRC; however, Boissière-Michot *et al* (37) reported that the density of CD4(+) TILs was increased in MSI-CRC compared with MSS-CRC. Pagès *et al* (38) revealed that 21% of patients with MSS tumors had high immunoscore densities of $CD3^+$ and $CD8^+$ TILs, similar to MSI tumors. The present study identified a subset of patients with pMMR-CRC with increased CD8(+) and CD4(+) TIL infiltration, as seen in dMMR-CRC. The aforementioned patients may benefit from immunotherapy with anti-PD-1/PD-L1 mAbs.

It is well known that regulatory T (Treg) cells express FOXP3 and suppress antitumor immune responses (39-43). In patients with CRC, the number of Treg cells (which suppress the activity of tumor antigen-specific T cells) is increased in the tumor microenvironment (44-46). Although several studies have evaluated the association between Treg cells and MSI status, the results were controversial (37,39-41,47). Differences in these studies may be attributed to the use of different methodologies, such as PCR and IHC, the types of lesions analyzed, including invasive front and surface of tumor, and the phenotypic diversity of Treg cells (48). Further investigation is required to clarify the association between Treg cells and MSI or MMR status in CRC.

A recent study demonstrated that in a large cohort of patients with CRC, those with high TMB accounted for 3% of MSS-CRC cases (49), indicating that a subset of patients with MSS-CRC with high TMB may benefit from treatment with ICIs. As the TMB status was not evaluated in the present study, it is currently unclear whether the subpopulation of patients with MSI-L/MSS-CRC with upregulated CD8 T effector and IFN-γ gene signatures corresponds with high mutation burden groups. Furthermore, conclusions about the responsiveness to ICI therapies cannot be drawn in the current study. Further investigations to clarify the association between TMB and immune profiling in MSI-L/MSS-CRC, as well as the clinical response in the identified subpopulation, are required.

While the MSS/TMB-high group may benefit from treatment with ICIs, there are several unresolved issues to accurately identify patients with MSS/TMB-high status. TMB analysis from whole exome sequencing is not widely available, as it is a time and cost intensive method (50,51). More importantly, it has recently been shown that patients with metastatic MSI-H/dMMR-CCR who exhibit primary resistance to ICIs may have been misdiagnosed (52). In other words, patients who had been initially diagnosed as MSI-H were actually MSS, indicating that MSI-testing is currently not accurate enough and not fully established. Furthermore, Wang *et al* (53) reported that patients with a polymerase ε-mutation present a hyper-mutated tumor phenotype caused by high frequent base substitution mutations, without necessarily giving rise to the short tandem repeat signature identified through MSI-H testing. In light of the aforementioned complexities, multiple predictive biomarkers are required to accurately select ICI responders with MSS/pMMR-CRC. The evaluation of CD8 T effector and IFN-γ gene signatures described in the current study may be used as part of a combination testing strategy for ICI responder identification.

The present study demonstrated that a subset of patients with upregulated CD8 T effector and IFN-γ gene signatures MSI-L/MSS- or pMMR-CRC may benefit from immunotherapy with anti-PD-1/PD-L1 mAbs.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in The Cancer Genome Atlas and were downloaded via cBioPortal (www.cbioportal.org).

Authors' contributions

KM and KK conceived and designed the study. HO and YN analyzed TCGA dataset. KS, LY, EE, WS, SF, HE, MS, TM, ZS and SO contributed to the acquisition of the patient samples. TK, KS, LY and EE performed and evaluated the IHC staining. TK, KM, HO, YN, WS, SF, HE, MS, TM, ZS and SO analyzed the patient data. TK, HO, KM and KK drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were conducted in accordance with the Helsinki Declaration and were approved by the responsible committee on human experimentation at Fukushima Medical University (Reference 2847). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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