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Metformin changes the immune microenvironment of colorectal cancer in patients with type 2 diabetes mellitus

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Abstract

Accumulating evidence suggests that metformin reduces the incidence and mortality of colorectal cancer (CRC). However, underlying mechanisms have not been fully clarified. The aim of this study was to examine the pathological characteristics of resected CRC from patients treated with metformin for type 2 diabetes mellitus (DM). In total, 267 patients with DM underwent curative colectomy for Stage I-III CRC and 53 (19.9%) patients had been treated medically including metformin. Pathological N-stage was significantly lower in metformin-treated patients ($P < .05$) with prolonged disease-free survival (DFS) ($P < .05$). Immunohistochemistry showed that the densities of CD3(+) and CD8(+) tumor-infiltrating lymphocytes (TILs) in the invasive front area were significantly higher in 40 patients treated with metformin compared with propensity score matched cases without metformin ($P < .05$). The density of tertiary lymphoid structures (TLS) in tumor stroma was markedly increased in metformin-treated patients ($P < .001$). In those tumors, there were more CD68(+) tumor-associated macrophages (TAM) infiltrated ($P < .05$), while the ratio of CD163(+) M2-phenotype was markedly reduced ($P < .001$). Stromal fibrosis tended to be suppressed by metformin intake ($P = .051$). These findings suggested that metformin drastically changes the characteristics of infiltrating immune cells in CRC and reprograms the tumor microenvironment from immunosuppressive to immunocompetent status, which may lead to suppression of microscopic tumor spread and improve the outcomes of patients with CRC and type 2 DM.

KEYWORDS

colorectal cancer, metformin, tertiary lymphoid structure, tumor-associated macrophage, tumor-infiltrating lymphocytes

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer globally and the fourth leading cause of mortality worldwide.[1] Type 2 diabetes mellitus (DM), the most common chronic and metabolism disease, is well known to increase the risk for development of various cancers and is associated with poor outcomes[2,3] including colorectal cancer (CRC).[4-6] Currently, it is considered that hyperglycemia, insulin resistance, and increased levels of insulin/insulin-like growth factors, as well as obesity and increased adipocytokines, play major roles in enhancing tumorigenesis and tumor progression.[2,3,7]

Metformin is an oral ant hyperglycemic agent that has been used as first-line treatment for type 2 DM for over 50 y. Numerous studies have suggested that metformin intake can significantly reduce the incidence of cancer development and mortality among patients with type 2 DM, although the degree of reduction varies among different types of cancer.[8-13] However, the mechanisms underlying how metformin exerts this anti-tumor effect is not fully understood. Preclinical studies have shown that metformin can reduce viability and proliferation of tumor cells, repress epithelial-mesenchymal transition, and increase chemosensitivity mainly through the inhibition of mTORC signaling.[14-16] Recently, however, metformin has been shown to lose its anti-tumor effects in severe combined immunodeficiency (SCID) mice, suggesting a critical role for host lymphocytes to exert these anti-tumor effects.[17,18] Metformin has also been shown to mediate the repolarization of macrophages from the M2 to the M1 phenotype in the tumor microenvironment, and this may lead to the inhibition of tumor growth in murine models.[19-21] More recently, metformin has been shown to downregulate programmed cell death receptor ligand-1 (PD-L1) in tumor cells that led to enhanced T-cell-mediated cytotoxicity.[22-24] These experimental results suggest that the anti-tumor properties of metformin are closely related to the host immune system. However, the mechanisms by which metformin modulates anti-tumor immune function in humans has not been satisfactorily investigated. In this study, the phenotypes of immune cells infiltrating human CRC tumors from patients who had and had not been treated with metformin were characterized by immunohistochemistry and the effect of metformin on the tumor immune microenvironment in human CRC was assessed.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue specimens

From January 2009 until June 2019, 1918 patients with CRC (Stage I-III) underwent curative colectomy in the Department of Surgery at Jichi Medical University Hospital. Among them, 267 patients (13.9%) also had type 2 DM and 53 patients (19.9%) were treated with medications including metformin at the time of surgery. They had taken metformin 500-1000 mg daily from 1 to 43 y (median = 10 y) and total dose was 183-15 695 g (median = 2738 g). In these patients, data for gender, age, disease name, operated day, surgical procedure, medical history, treatment of diabetes, preoperative laboratory, pathological results (histological type, depth of tumor, nodal metastasis, vascular invasion, lymphatic invasion) and outcome were extracted from an electronic database with written informed consent. This study was approved by the ethics committee of the Jichi University Hospital (approval no. clinic19-190) and was conducted in accordance with the guiding principles of the Declaration of Helsinki.

2.2 | Antibodies and reagents

Monoclonal antibodies (Abs) to CD3(60347-1-Ig, clone 2E9G7)and CD8α (66868-1-Ig, clone 1G2B10 were purchased from Proteintech Group, (Rosemont, USA) and to CD68 (ab955, clone KP1), CD163 (ab156769, clone OTI2G12) and CD20 (ab9475, clone L26) were from Abcam (Cambridge, MA). Signal enhancer HIKARI for Immunostain Solution B, antibody dilution buffer, HistoVT One (10×, pH 7.0, 06380-05) and blocking solution One Histo (06349-64) were purchased from Nacalai Tesuque (Kyoto, Japan).

2.3 | Histopathology and immunohistochemistry

The tissue sections from surgically resected specimens were available for immunohistochemistry studies in 40/53 patients treated with metformin (metformin(+)). Samples from other 40 patients without metformin intake (metformin(–)) were selected from the remaining 237 patients by propensity score matching method and used as a control group in immunostaining. All specimens were fixed in formalin, embedded in paraffin, cut into 4-μm thick sections and used for immunohistochemistry (IHC) as well as hematoxylin-eosin (HE) and Masson-Trichrome staining.

Immunohistochemical staining was performed using the DAKO REAL™ Envision™ Detection system (Glostrup, Denmark). Briefly, after deparaffinization in xylene and rehydration in a graded series of ethanol baths, the sections were washed with distilled water for 10 min. For antigen retrieval, the sections were processed by heating at 90°C in HistoVT One for 30 min. Endogenous peroxidases were blocked using 0.3% hydrogen peroxide for 30 min. After washing in phosphate-buffered saline (PBS), a nonspecific staining blocking agent (Blocking One Histo) was used to prevent nonspecific binding for 10 min. The sections were then incubated with primary antibodies for CD3 (1:100 dilution), CD8α (1:4000), CD20 (1:1000), CD68 (1:200), and CD163 (1:300) for 60 min at room temperature. The sections were thoroughly washed with PBS and incubated with DAKO REAL™ EnVision™/HRP, Rabbit/Mouse (code K5007, DakoCytomation, Denmark), and the primary antibody binding visualized using a the DAKO Envision kit according to the manufacturer's instructions and counterstained with Meyer's hematoxylin.

In evaluation of the density of immune cells, positive cells were counted in 5 randomly selected fields at the invasive front area by under ×400 light microscope. Analysis was blinded with respect to clinical outcomes by 2 investigators. In IHC of continuous sections,

tertiary lymphoid structures (TLS) were defined as a cluster of CD8(+) T-cell associated with CD20(+) B cells, which often formed germinal centers (GC) (Figure 5). The number of TLS with or without a GC was counted in 5 randomly selected areas in stroma by an expert investigator who was blinded to clinical data. In evaluation of fibrosis, staining intensities of fibrosis in tumor stroma with Masson-Trichrome staining were independently scored from Grades 0 to 3 (Figure 7A) at low magnification field by 4 different evaluators who were unaware of the clinical findings and the average of their scores were calculated.

2.4 | Statistical analysis

Statistical analyses were performed using GraphPad Prism 8. Statistical differences in clinical and pathological factors were evaluated with the Mann-Whitney *U* test. Correlation between cell densities was analyzed with Pearson simple linear regression analysis. Disease-free survival (DFS) was calculated using the Kaplan-Meier method and differences were evaluated using the log-rank test. In all tests, the standard for a significant difference was set at $P < .05$.

3 | RESULTS

3.1 | Metformin use is associated with fewer nodal metastases

Table 1 shows the clinical and pathological characteristics of the patients with CRC and type 2 DM who underwent curative surgery. Although the 53 metformin(+) patients were younger than the 214 metformin(−) patients, there were no significant differences between the 2 groups for gender, tumor site, histological type, and hemoglobin A_{1c} (HbA_{1c}) levels. Pathological T-stage and N-stage as well as P-stage tended to be lower in metformin(+) patients with a significant difference in pN category ($P = .048$). The rates of nodal metastases in patients with pT1, pT2, pT3 and pT4 lesions in metformin(−) patients were 4% (2/50), 22% (7/32), 46% (33/72), and 53% (32/60), respectively. In comparison, those in metformin(+) patients were 0% (0/14), 11% (1/9), 35% (8/23), and 29% (2/7). The number of metastatic nodes in the metformin(+) group was significantly lower than those in their metformin(−) counterparts ($P = .045$) with a similar trend at every pT stage (Figure 1).

3.2 | Patient outcomes

With a median follow-up of 3.6 y, recurrences occurred in 38/214 metformin(−) patients (17.8%). In comparison, only 3/53 metformin(+) patients (5.6%) recurred over a similar follow-up period (median = 3.5 y), and the DFS of metformin(+) patients was significantly longer in than their metformin(−) counterparts ($P = .045$) (Figure 2A). However, metformin intake did not have an independent correlation with DFS in multivariate Cox regression analysis (Table 2).

TABLE 1 Patients with colorectal cancer (CRC) with or without metformin use

Clinical and pathological factors	Metformin use		
	Metformin (53)	No metformin (214)	*P*-value
Age	66 (42-79)	70 (42-91)	<.05
Gender			
Male	38	145	.75
Female	15	69	
Tumor site			
Right	17	74	.87
Left	36	140	
Histological type			
Tub/pap	51	205	.99
Por/muc	2	9	
pT category			
t1	14	50	.13
t2	9	32	
t3	23	72	
t4	7	60	
pN category			
n0	42	140	.048
n1	9	55	
n2	2	17	
n3	0	2	
P-stage			
I	21	70	.10
II	21	70	
III	11	74	
p-lymphatic invasion			
Yes	23	104	.49
No	29	108	
Unknown	1	2	
p-vascular invasion			
Yes	37	152	.69
No	15	60	
Unknown	1	2	
Hemoglobin A_{1c}	6.9 (5.3-11.3)	6.7 (5.3-9.4)	.11

3.3 | Metformin changes the phenotype of tumor-infiltrating lymphocytes (TILs) and macrophages in CRC

As metformin modulates various immunological components including lymphocytes, macrophages, and cytokines, we examined the phenotypes of T cells (Figure 3, left panel) and macrophages (Figure 3, right panel) infiltrating resected tumors with immunohistochemistry using mAbs to CD3, CD8 and CD163, CD68, respectively. Among the 53 metformin(+) patients, 40 tumors could be sufficiently evaluated with immunohistochemical staining. Table 3 shows the profiles of the 40 tumors and 40 tumors from metformin(−) patients selected by a

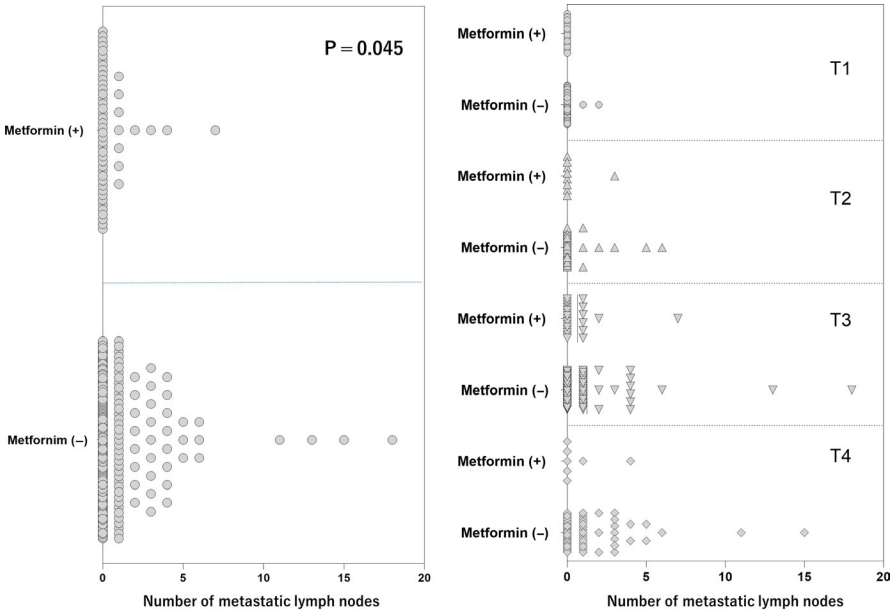

FIGURE 1 Comparison of the depth of invasion (T-stage) and the number of metastatic lymph nodes in cases with (n = 53) or without (n = 214) metformin intake. *P*-value was calculated using Mann-Whitney *U* test

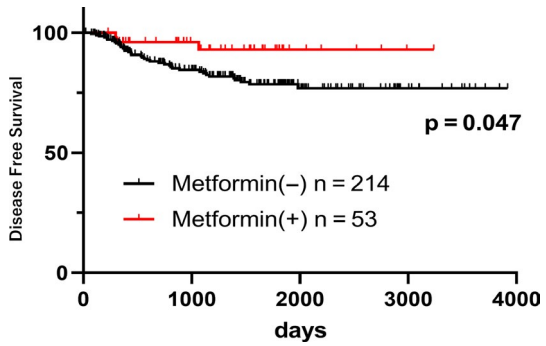

FIGURE 2 A, Disease-free survival (DFS) after surgery in patients with (n = 53) or without (n = 214) metformin intake were evaluated using the Kaplan-Meier method and *P*-value was calculated using the log-rank test

propensity score matching method. As shown in Figure 4A, the median (M) of the densities of CD3(+) T cells infiltrating metformin(+) tumors was 147 (78-389)/high power field (HPF) and was significantly greater than tumors from metformin(–) patients (M = 121, 30-229/HPF, $P < .05$). However, the density of tumor-infiltrating CD8(+) T cells was significantly greater in metformin(+) tumors (M = 100 [57-320]/HPF vs M = 60 [14-182]/HPF, $P < .01$), and the CD8(+)/CD3(+) ratios were significantly higher in metformin(+) patients (M = 74% [36%-86%] vs M = 50% [33%-86%], $P < .001$) (Figure 4B,C).

The density of CD68(+) macrophages was also higher in metformin(+) tumors (M = 107 [67-161]/HPF vs M = 82 [56-158]/HPF, $P < .01$), whereas the number of CD163(+) M2 macrophages in metformin(+) tumors tended to be lower than in their counterparts (M = 56 [27-96]/HPF vs M = 67 [33-125]/HPF, $P = .10$) (Figure 4D,E). Thus, the ratios of M2 macrophages calculated by CD163(+)/CD68(+) cells were M = 57% (33%-79%) in metformin(+) tumors and this was significantly less than those in metformin(–) tumors (M = 77% [58%-85%], $P < .001$) (Figure 4F).

Interestingly, a clear inverse correlation was observed between CD8(+)/CD3(+) and CD163(+)/CD68(+) ratios in those 80 patients ($r = 0.60$, $P < .001$) (Figure S1). However, the distribution of each patient in this plot showed sharp contrast with (red circles) or without (black circles) metformin use.

3.4 | Metformin use increases the number of TLS associated with T-cell infiltration in CRC

TLS are ectopic lymph node-like structures that often develop in tumors due to an immune response against tumor antigens. As

TABLE 2 Univariate and multivariate analyses of the correlation between variables and disease-free survival of the patients with colorectal cancer

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age	1.019 (0.983-1.055)	.302		
Gender (Male/Female)	1.039 (0.530-2.037)	.911		
Pathological T-stage	2.590 (1.729-3.879)	<.001	2.095 (1.372-3.199)	<.001
Pathological N-stage	2.948 (2.072-4.194)	<.001	2.124 (1.451-3.109)	<.001
Adjuvant therapy	1.811 (0.907-3.615)	.092		
Metformin intake	0.321 (0.099-1.040)	.058	0.498 (0.152-1.640)	.25

Abbreviations: CI, confidence interval; HR, hazard ratio.

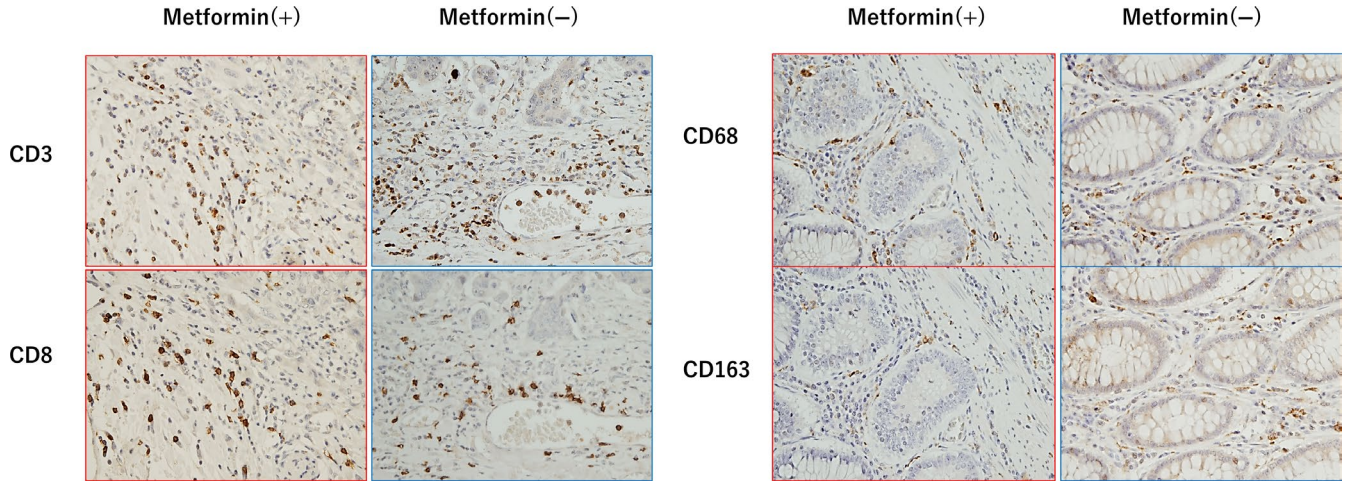

FIGURE 3 Representative images of immunostaining of tumor-infiltrating lymphocytes (TIL) and tumor-associated macrophages (TAM) in metformin-treated and non-treated colorectal cancer (CRC) (original magnification ×400)

TABLE 3 Characteristics of colorectal cancer in patients with or without metformin use evaluated by immunohistochemistry

	Metformin(+) (n = 40)	Metformin(−) (n = 40)	*P*-value
Tumor site			
Cecum/Ascending/ Transverse	16 (40.0%)	18 (45.0%)	>.99
Descending/Sigmoid	9 (22.5%)	8 (20.0%)	
Rectum	15 (37.5%)	14 (35.0%)	
Histological type			
Tub1/tub2/pap/	39 (97.5%)	39 (97.5%)	>.99
Muc/por	1 (2.5%)	1 (2.5%)	
pT category			
t1	6 (15.0%)	12 (30.0%)	.83
t2	8 (20.0%)	5 (12.5%)	
t3	19 (47.5%)	12 (30.0%)	
t4	7 (17.5%)	11 (27.5%)	
pN category			
n0	32 (80.0%)	32 (80.0%)	>.99
n1	8 (20.0%)	8 (20.0%)	
P-stage			
I	13 (32.5%)	14 (35.0%)	>.99
II	19 (47.5%)	18 (45.0%)	
III	8 (20.0%)	8 (20.0%)	

shown in Figure 5, many TLS were detected as aggregated CD20(+) B cells and CD8(+) T cells and most of them formed GC in the stromal area of the CRC from metformin(+) patients. The total numbers of TLS in 5 randomly selected fields were significantly greater in metformin(+) CRC than metformin(−) CRC (M = 10, 1-21 vs M = 5, 0-19, $P < .001$) (Figure 6A). Figure 6B shows the same trend in the number of TLS with GC (M = 2.5, 0-13 vs M = 1, 0-12, $P < .05$). The TLS density had a positive correlation with the CD8(+) density ($r = 0.36$, $P = .0011$) as well as CD3(+) ($r = 0.30$, $P = .0061$) T cells in all patients (Figure 6C,D). In these 80 patients with or without metformin intake, the DFS of the patients with high density of TLS tended to be better than that of patients with a low density of TLS ($P = .09$) (Figure S2).

3.5 | Metformin intake was associated with reduced fibrosis in CRC

The degree of stromal fibrosis in CRC was objectively evaluated by staining collagen fibers with Masson-Trichrome. As shown in Figure 7, the average fibrotic scores of metformin(+) CRC were lower than those from metformin(−) tumors with a marginally significant difference ($P = .051$).

4 | DISCUSSION

Epidemiological studies suggested that metformin not only reduces the risk of developing CRC but also may improve the outcome of patients with DM and CRC, especially for patients who have undergone curative surgery for stage II and stage III tumors,[25-31] although a large population-based study did not support a significant protective association between metformin and mortality in patients with CRC.[32] Many experimental studies using animal models have suggested that metformin directly suppresses the growth and metastasis of tumor cells.[14-16] However, the pathological features of tumors in patients treated with metformin have not been well characterized especially in humans, and the mechanisms leading to improved survival still remain unknown.

In this study, we confirmed that the metformin improves the DFS after curative surgery for CRC in 267 patients with type 2 DM. We found that pathological T-stage, N-stage and stage of CRC tumors in metformin(+) patients tended to be less advanced compared with

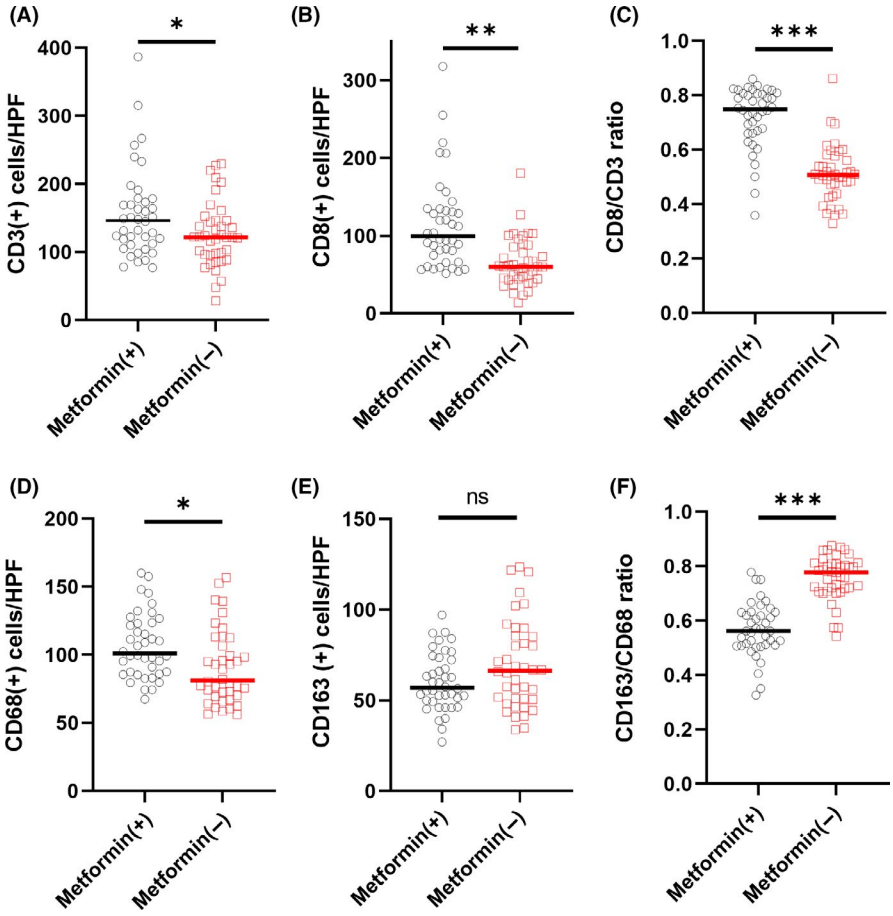

FIGURE 4 A-C, Comparison of the density of CD3(+) and CD8(+) tumor-infiltrating lymphocytes (TILs) and the ratio of CD8(+)/CD3(+) in invasive front of colorectal cancer (CRC) between metformin-treated and non-treated cases. D-F, Comparison of tumor-associated macrophage (TAMs) and the ratio of CD163(+)/CD68(+) in the same area of the CRC. *P*-values were calculated using Mann-Whitney *U* test and Pearson simple linear regression analysis. **P* < .05, ***P* < .01, ****P* < .001. HPF, high power field

tumors in the metformin(–) group. The rates and number of nodal metastases were significantly lower in metformin(+) patients. The same significant association was not observed in previous studies in which N-stage was classified based on clinical findings.[26-28] However, as metformin intake did not show an independent correlation with DFS in multivariate analysis, the effects of metformin on patient outcomes in this series are suggested to be caused by the differences in microscopic cancer spread at surgery.

Immunohistochemical studies clearly show that the number of CD3(+) TILs, especially the CD8(+) phenotype, was increased in metformin(+) CRC. Recent studies have suggested that metformin affects various immunological components in both humans and animals. Eikawa et al demonstrated that metformin increases the number of CD8(+) TILs and enhances the efficacy of T-cell-mediated tumor cell lysis in a murine model.[17] Recent studies have shown that the number of CD8(+) TILs in human head and neck squamous cell carcinoma tumors (HNSCC)[33] and non-small-cell lung cell tumors[34] are increased in metformin-treated patients, and is consistent with these results.

The total number of TLS as well as TLS with GC in tumor stroma was greatly increased in the metformin(+) CRC. TLS are ectopic

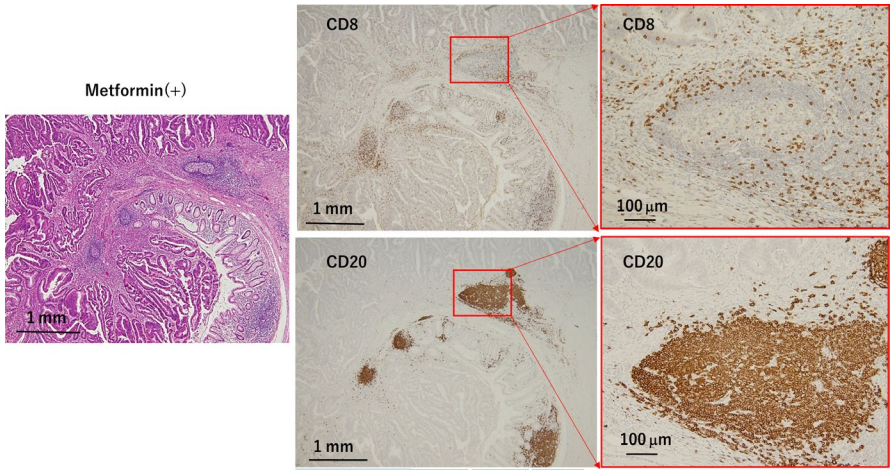

FIGURE 5 Representative image tertiary lymphoid structures (TLS) with germinal centers in colorectal cancer with metformin intake (HE and immunostaining of CD20(+) B cells and CD8(+) T cells in continuous tissue sections). In these TLS, clusters of CD20(+) B cells were surrounded by CD8(+) T cells to form germinal centers (GC)

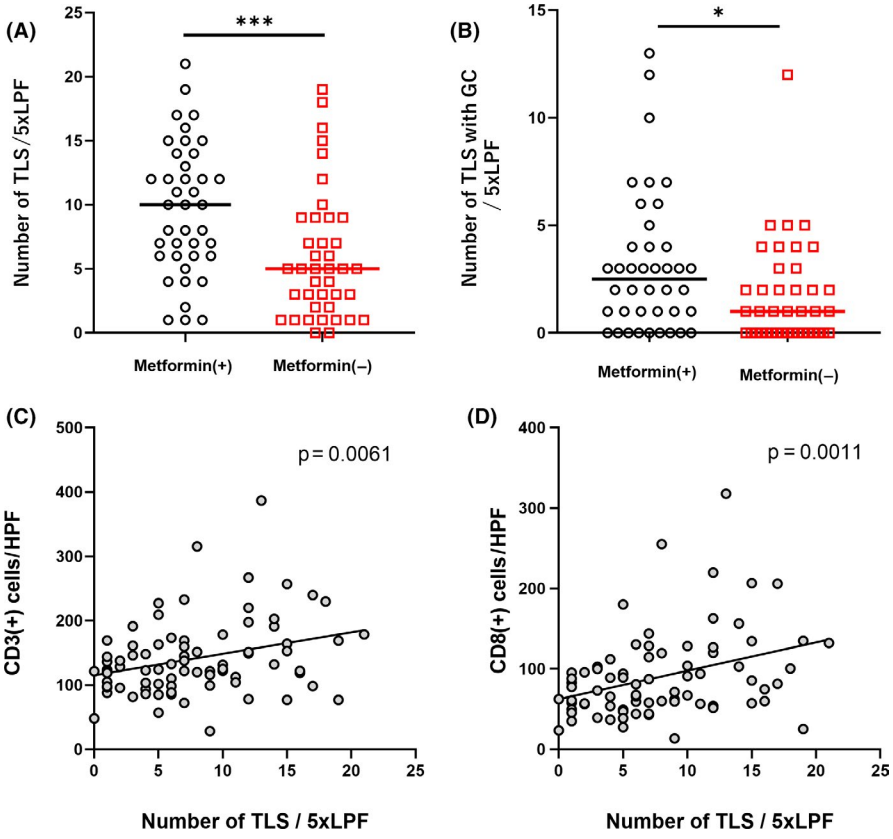

FIGURE 6 Numbers of total tertiary lymphoid structures (TLS) (A) and TLS with germinal center (GC) formation (B) in 5 randomly selected low power fields (LPF) in patients with colorectal cancer with or without metformin treatment. C, D, Correlation between the density of stromal TLS and those of CD3(+), CD8(+) TILs in all patients. *P*-values were calculated using Mann-Whitney *U* test and Pearson simple linear regression analysis. $^{*}P < .05$, $^{***}P < .001$. HPF, high power field

lymphoid organs that develop in peripheral tissues with chronic inflammation, including cancers, and exist in different maturation status in a tumor, culminating in GC formation.[35,36] Recent studies have suggested that TLSs represent privileged sites for generation of effector T cells, memory B cells and antibodies against tumor antigens and that the density of TLS in the tumor microenvironment (TME) is associated with favorable outcomes of patients with various solid malignancies including CRC.[36-38] TLS density has been shown to correlate with density of CD4(+) and CD8(+) TILs in early-stage NSCLC[39] as well as CRC.[37] The data in this study were mostly consistent with previous results and suggest that metformin may increase TILs with anti-tumor properties through the induction of TLS, resulting in the limited lymphatic spread of tumor cells in patients with CRC treated with metformin.

In general, tumor-associated macrophages (TAM) are known to be polarized to the immunosuppressive M2 phenotype and to

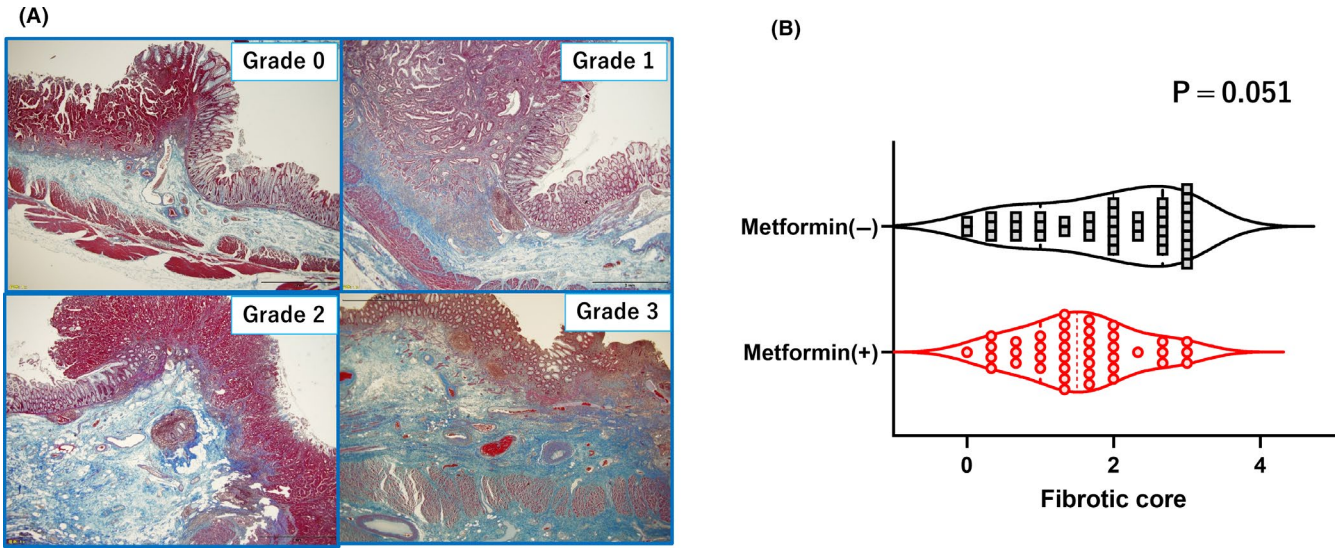

FIGURE 7 Degree of stromal fibrosis in colorectal cancer was determined from Grades 0 to 3 (A) by 4 different evaluators with Masson-Trichrome staining (original magnification ×400) and average fibrotic scores were compared between metformin-treated and non-treated tumors (B). *P*-value was calculated using the Mann-Whitney *U* test

facilitate a protumorigenic function by producing anti-inflammatory cytokines or angiogenic factors.[40,41] Recent studies, however, have shown that metformin can change M2-like macrophages to the M1-like phenotype and stimulates M1-related and inhibits M2-related cytokine production both in murine[20,21,42] and human systems.[19] Yin et al have shown that metformin reduced M2-type TAM in HNSCC, which causes sensitization to gefitinib treatment.[43] In the present study, the rate of M2 macrophages defined by CD163(+)/CD68(+) expression was markedly reduced in the metformin(+) group. Together, those results suggest that metformin switches the balance of TAM in favor of M2 to an M1 predominant state even in those cancers, which may suppress tumor progression and contribute to a favorable outcome of those patients. In fact, a recent study has shown that the ratio of CD163(+)/CD68(+) macrophages in tumor tissue is an independent prognostic factor for survival of patients with CRC.[44]

Another interesting finding is that CRC in metformin(+) group is less fibrotic. Intratumor fibrosis results from the deposition of collagen matrix mainly produced by cancer associated fibroblasts (CAF) and has a critical influence on the metastatic behavior of tumor cells.[45,46] Metformin has been shown to prevent fibrosis in various organs in preclinical models mainly through the AMPK-mediated suppression of TGF-β production.[47-50] As TGF-β is known to be produced by M2-type macrophages, the present study suggests a possibility that functional change of TAM is involved in the reduced stromal fibrosis in metformin-treated CRC.

Metformin increases the number of TLS and CD3(+) CD8(+) TILs, reduces the rate of M2-type TAMs, and promotes stromal fibrosis in human CRC, which may change the tumor microenvironment from immunosuppressive to an immunocompetent status. Increasing evidence has suggested that the anti-tumor properties of metformin are largely dependent on the host immune system.[17-21] In this series, the total dose of metformin as well as HbA_{1c} levels did not show significant correlation with stage, patient outcome, and densities of immune infiltrates, presumably because of the small sample size. Although a further study with larger number of cases is necessary, the present results are in line with this concept and suggest that the modulation of immune infiltrates at tumor sites may be a key mechanism to explain the positive impact of metformin on the outcome of the patients with cancer. As clinical responses to cytotoxic drugs, radiation or immune checkpoint inhibitors are largely dependent on the tumor immune microenvironment,[51,52] combination with metformin may effectively enhance the response to various anti-cancer treatments.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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