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

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Funding information
Japan Society for the Promotion of Science, Grant/Award Number: 19K09225

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 outcomes2,3 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 antihyperglycemic 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.3,11,12 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-lg, clone 2E9G7) and CD8 (66868-1-lg, clone 1G2B10 were purchased from Proteintech Group, (Rosemont, USA) and to CD68 (ab955, clone KP1), CD163 (ab156769, clone OT1ZG12) and CD20 (ab9475, clone L26) were from Abcam (Cambridge, MA). Signal enhancer HIKARI for Immunostain Solution B, antibody dilution buffer, HistoVT One (10x, 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), CD8 (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 investiga-
tor who was blinded to clinical data. In evaluation of fibrosis, staining
intensities of fibrosis in tumor stroma with Masson-Trichrome stain-
ing 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 evalu-
ated with the Mann-Whitney U test. Correlation between cell den-
sities 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 pa-
tients with CRC and type 2 DM who underwent curative surgery. Al-
though the 53 metformin(+) patients were younger than the 214
metformin(-) patients, there were no significant differences be-
tween the 2 groups for gender, tumor site, histological type, and
hemoglobin A1c (HbA1c) 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 met-
formin(-) 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 simi-
lar 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 met-
formin(+) patients (5.6%) recurred over a similar follow-up period
(median = 3.5 y), and the DFS of metformin(+) patients was sig-
ificantly 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).

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

As metformin modulates various immunological components includ-
ing lymphocytes, macrophages, and cytokines, we examined the phe-
notypes 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

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

| Clinical and pathological factors | Metformin use | P-value |
|---------------------------------|--------------|---------|
| Age                             |              |         |
| Metformin (53)                  | 66 (42-79)   | .05     |
| No metformin (214)             | 70 (42-91)   |         |
| Gender                          |              | .75     |
| Male                            | 38           |         |
| Female                          | 15           |         |
| Tumor site                      |              | .87     |
| Right                           | 17           |         |
| Left                            | 36           |         |
| Histological type               |              | .99     |
| Tub/pap                         | 51           |         |
| Por/muc                         | 2            |         |
| pT category                     |              | .13     |
| t1                              | 14           |         |
| t2                              | 9            |         |
| t3                              | 23           |         |
| t4                              | 7            |         |
| pN category                     |              | .048    |
| n0                              | 42           |         |
| n1                              | 9            |         |
| n2                              | 2            |         |
| n3                              | 0            |         |
| P-stage                         |              | .10     |
| I                               | 21           |         |
| II                              | 21           |         |
| III                             | 11           |         |
| p-lymphatic invasion            |              | .49     |
| Yes                             | 23           |         |
| No                              | 29           |         |
| Unknown                         | 1            |         |
| p-vascular invasion             |              | .69     |
| Yes                             | 37           |         |
| No                              | 15           |         |
| Unknown                         | 1            |         |
| Hemoglobin A1c                  |              | .11     |
| 6.9 (5.3-11.3)                  | 6.7 (5.3-9.4) |         |
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

| Variables       | Univariate analysis | Multivariate analysis |
|-----------------|---------------------|-----------------------|
|                 | 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.
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, although a large population-based study did not support a significant protective association between metformin and mortality in patients with CRC. Many experimental studies using animal models have suggested that metformin directly suppresses the growth and metastasis of tumor cells. 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
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 tumors34 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...
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. 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. TLS density has been shown to correlate with density of CD4(+) and CD8(+) TILs in early-stage NSCLC as well as CRC. 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 6](image6.png)

**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(+) and 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

![Figure 7](image7.png)

**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. 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 and human systems. Yin et al have shown that metformin reduced M2-type TAM in HNSCC, which causes sensitization to gefitinib treatment. 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.

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. Metformin has been shown to prevent fibrosis in various organs in preclinical models mainly through the AMPK-mediated suppression of TGF-β production. 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. In this series, the total dose of metformin as well as HbA1c 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, combination with metformin may effectively enhance the response to various anti-cancer treatments.

ACKNOWLEDGMENTS

This work was supported by the Japan Society for the Promotion of Science (19K09225). We thank Professor T. Niki for his advice on the evaluation of immunohistochemistry. We also thank Ms J. Shinohara, H. Hatakeyama, N. Nishiaki and I. Nieda for technical and clerical work.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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