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# Pathological Complete Remission of Liver Metastases Correlates With Elimination of Tumor-infiltrating Tregs in Gastric Cancer

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**Abstract.** *Background/Aim: Tumor-infiltrating Foxp3$^+$ regulatory T-cells (Ti-Tregs) promote tumor progression and contribute to poor prognosis in gastric cancer, but the relationship between Ti-Tregs and response to chemotherapy for liver metastases from gastric cancer (LMGC) is unclear. We estimated the correlation between pathological response to chemotherapy and Ti-Tregs in LMGC. Patients and Methods: Ti-Tregs were analyzed with immunohistochemistry as CD3$^+$ Foxp3$^+$ cells in patients with synchronous LMGC. Results: Of 53 patients with LMGC, 49 received chemotherapy as initial treatment and 10 underwent R0 resection. LMGC disappeared pathologically in 5 resected cases despite radiologically residual disease. Ti-Tregs were found frequently in residual LMGC and primary lesions but rarely in tumor scar tissue. There was no relationship between frequency of CD8$^+$ cells and pathological response. Conclusion: Marked reduction in Ti-Tregs correlates with pathological complete remission of LMGC. Ti-Tregs may be a biomarker to predict the effects of chemotherapy when used in combination with radiological findings.*

Liver metastases are common in gastric cancer and contribute to poor prognosis (1, 2). Although systemic chemotherapy is the most important treatment for liver metastases from gastric cancer (LMGC), survival is longer with additional surgical intervention than with systemic chemotherapy alone (1, 3). It was recently suggested that conversion surgery after chemotherapy improves clinical outcomes in patients with resectable LMGC (4). Marked reduction in tumor size can be achieved by systemic chemotherapy in some cases. However, complete remission is difficult to diagnose radiologically.

Foxp3$^+$ regulatory T-cells (Tregs) are known to induce immune tolerance by immunosuppression of other immune cells (5, 6) and to promote tumor growth in gastric cancer (7). Increasing tumor-infiltrating Tregs (Ti-Tregs) is reported to be a poor prognostic factor in the primary gastric lesion (8, 9). In breast and esophageal cancers, a correlation has been reported between complete pathological remission after neoadjuvant chemotherapy or chemoradiation therapy and elimination of Ti-Tregs (10, 11). However, such relationships have not yet been fully investigated in primary and metastatic gastric lesions.

In this study, we used immunohistochemistry to analyze the number of Ti-Tregs in patients who received chemotherapy followed by gastrectomy and hepatectomy in order to clarify the relationship between Ti-Tregs and pathological efficacy. We found that Ti-Tregs may be a useful biomarker to predict the efficacy of chemotherapy against LMGC.

## Patients and Methods

*Patients, diagnosis, and treatment.* Fifty-three patients with synchronous LMGC but no other non-curative features were treated between 2010 and 2019 at Hyogo College of Medicine. Upper gastrointestinal endoscopy was performed in all cases and the diagnosis of gastric cancer was confirmed histologically. Histopathological examination was performed by two pathologists. Liver metastases were diagnosed on contrast-enhanced computed tomography and contrast-enhanced ultrasonography performed by two radiologists. $^{18}$Fluorodeoxyglucose positron emission tomography and gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid magnetic resonance imaging were performed as necessary. In 1 patient, LMGC were detected on the liver surface by staging laparoscopy. Needle biopsy for LMGC was not performed.

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*Key Words:* Liver metastases from gastric cancer, tumor-infiltrating Foxp3+ regulatory T-cells, pathological response to chemotherapy.

Table I. *Clinicopathological features of patients with synchronous LMGC who underwent R0 resection after chemotherapy.*

| Case | Age | Gender | Liver metastases | | Pathological type | HER2 | Line/regimen/kurr | Clinical response to chemotherapy (RECIST) | ypH | Histological effect (grade) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Number | Maximum diameter | | | | | | |
| #1 | 68 | F | 1 | 18 | Undifferentiated | Negative | First/SP/2 | PR | 1 | 1a |
| #2 | 62 | M | 1 | 14 | Differentiated | Negative | First/SP/2 | PR | 0 | 3 |
| #3 | 64 | M | 1 | 34 | Differentiated | Positive | First/XP+HER/4 | PR | 1 | 1b |
| #4 | 77 | M | 1 | 25 | Differentiated | Negative | First/SP/2 | PR | 0 | 1a |
| #5 | 66 | M | 1 | 17 | Undifferentiated | Negative | Third/CPT/6 | PR (3rd line) | 0 | 1b |
| #6 | 60 | M | 2 | 6 | Differentiated | Negative | First/SP/4 | PR | 0 | 1a |
| #7 | 70 | M | 4 | 34 | Differentiated | Positive | First/XP+HER/1<br>XELOX+HER/12 | PR | 1 | 1a |
| #8 | 56 | M | 5 | 10 | Differentiated | Positive | First/XP+HER/7<br>Second/DCS/2 | PR | 1 | 1a |
| #9 | 51 | M | 6 | 90 | Differentiated | Positive | First/SOX+HER/8<br>S-1+HER/5 | PR | 0 | 3 |
| #10 | 68 | M | 13 | 86 | Undifferentiated | Negative | Second/PTX+RAM/21 | PR (2nd line) | 1 | 2 |

Chemotherapy was administered according to the 2018 Japanese gastric cancer treatment guidelines (12). The clinical and pathological response to chemotherapy was estimated using the RECIST (Response Evaluation Criteria in Solid Tumors) criteria and the histological evaluation criteria for preoperative chemotherapy in the 15th edition of the Japanese Classification of Gastric Carcinoma (13). The study was approved by the institutional review board of Hyogo Medical College of Medicine (approval number 2754). All patients provided written informed consent.

*Immunohistochemical labeling*. Formalin-fixed, paraffin-embedded tissue samples from the primary lesion and liver metastases were cut into 5-μm thick sections and deparaffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was carried out by heating the slides for 27 min in a microwave oven with citrate buffer (pH 6.0) at 98˚C. After blocking with 3% bovine serum albumin in phosphate-buffered saline, the slides were incubated for 2 h at room temperature with the following primary antibodies: mouse monoclonal antibody for Foxp3 (236A/E7, dilution 1:50; eBioscience, San Diego, CA, USA), rabbit monoclonal antibody for CD3 (ab5690, 1:100; Abcam, Cambridge, UK), and mouse monoclonal antibody for CD8α (ab17147, 1:25; Abcam). After washing with 0.1% bovine serum albumin in phosphate-buffered saline, the slides were stained with goat anti-mouse IgG H&L Alexa Fluor 488 (ab150114, 1:500; Abcam) and donkey anti-rabbit IgG H&L Alexa Fluor 647 (ab150075, 1:1000; Abcam) secondary antibodies for 1 h. The slides were covered with a coverslip and ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) after staining. The stained sections were observed under a confocal microscope (LSM 780; Carl Zeiss, Oberkochen, Germany).

Foxp3 was expressed in the nucleus and CD3 and CD8 were expressed on the cell membrane. $Foxp3^+$ Tregs were defined as cells that were positive for both CD3 and Foxp3. The populations of $CD3^+$ $Foxp3^+$ cells and $CD8^+$ cells were estimated according to the percentages of $CD3^+$ $Foxp3^+$ cells in $CD3^+$ cells and $CD8^+$ cells in $CD3^+$ plus $CD8^+$ cells and scored as follows: 0, 0-2%; 1, 3-10%; 2, 11-30%; 3, 31-50%; 4, 51-100%.

*Statistical analyses*. Data are shown as the mean and standard deviation. The Mann-Whitney *U*-test was used for comparisons. All statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA). *p*-Values less than 0.05 were considered statistically significant.

## Results

*Clinical efficacy of chemotherapy and pathological diagnosis of LMGC*. Forty-nine (92.3%) of the 53 cases of synchronous LMGC received systemic chemotherapy and the remaining 4 (7.7%) underwent gastrectomy and partial hepatectomy as initial treatment because of detection of a liver tumor during gastrectomy or difficulty distinguishing between hepatocellular carcinoma and LMGC. Of the 49 patients, 10 (20.4%) underwent R0 resection that included gastrectomy and hepatectomy after chemotherapy.

Table I shows the clinicopathological features of patients with synchronous LMGC who underwent R0 resection after chemotherapy. The liver metastases resected after chemotherapy were solitary and small. HER2 expression (score 3+ or 2+; FISH-positive) was detected in the primary lesion in 4 patients, all of whom were treated with chemotherapy combined with trastuzumab. Seven patients underwent surgery after first-line chemotherapy and the others after second-line or third-line chemotherapy. Partial response was achieved in all patients who underwent resection. In patient #10, who had multiple (n=13) liver metastases, second-line chemotherapy (ramucirumab plus paclitaxel) resulted in partial response and R0 resection was achieved by a liver-first two-stage operation.

Pathological complete remission in the resected liver tumors (ypH0) was achieved in 5 (50%) of the 10 patients

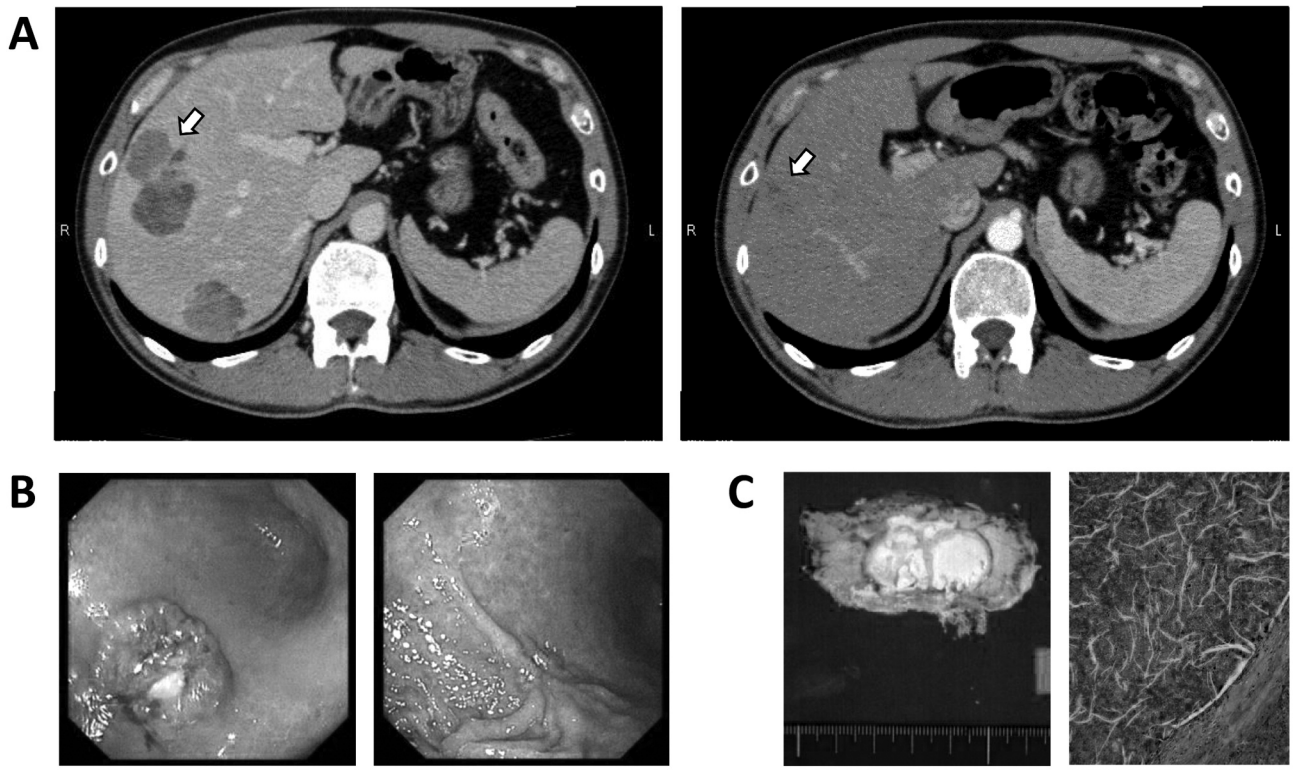


Figure 1. *Clinical and pathological findings after effective chemotherapy for liver metastases from gastric cancer. (A) In case 9, multiple liver metastases were observed on contrast-enhanced computed tomography images before (left) and after (right) systemic chemotherapy. (B) Endoscopic images of the primary gastric lesion before (left) and after (right) chemotherapy. (C) Findings on macroscopic and microscopic examination of the liver metastases.*

who underwent surgery. In particular, there was no tumor scar in the resected liver specimen in patient #2 and 2 patients (case #2 and #9) achieved pathological complete remission of the primary lesion. Patient #9 was diagnosed with HER2-positive gastric cancer with multiple liver metastases. Systemic chemotherapy plus anti-HER2 monoclonal antibody achieved a dramatic size reduction in both the liver metastases (Figure 1A) and the primary lesion (Figure 1B). Distal gastrectomy and partial hepatectomy were performed after chemotherapy was administered for more than 1 year. There were no histological residual lesions in the resected specimens (Figure 1C). The metastatic lesions in the liver sometimes disappeared pathologically when chemotherapy for the gastric cancer was effective.

*Ti-Tregs were almost completely eliminated in the tumor scar of LMGC after chemotherapy.* The number of $CD3^+$ $Foxp3^+$ cells in LMGC and primary lesions were analyzed to determine the correlation between histological chemotherapeutic efficacy and number of Ti-Tregs. Foxp3 expression has been detected in lymphocytes and cancer cells in gastric cancer (14). We analyzed CD3 and Foxp3 expression to estimate Foxp3 expression in $CD3^+$ T cells. $CD3^+$ cells and $CD3^+$ $Foxp3^+$ cells were located at the invasive front of cancer tissue (Figure 2A). $CD3^+$ $Foxp3^+$ cells were often detected in LMGC in patients who did not undergo induction chemotherapy and had residual cancer after chemotherapy. Otherwise, these cells were almost completely not detectable in the tumor scar after chemotherapy (ypH0) (Figure 2B). The percentage of $CD3^+$ $Foxp3^+$ cells in $CD3^+$ cells was small in the ypH0 group compared with no chemotherapy and ypH1 groups (Figure 2C). In patient 10, some of the metastases disappeared histologically. Although $CD3^+$ $Foxp3^+$ cells were detected in residual liver metastases, they were almost completely absent at sites of histological remission (Figure 2D). $CD3^+$ $Foxp3^+$ cells were detected more frequently in the primary gastric lesion than in scar tissue with pathological complete remission (Figure 2C). In contrast, $CD8^+$ cells were detected at the invasive front and in tumor tissues (Figure 3A). There was no difference in the percentage of $CD8^+$ cells in $CD8^+$ cells and/or $CD3^+$ cells between these three groups (Figure 3B). Immunoregulatory $Foxp3^+$ T-cells were almost completely absent in lesions that disappeared pathologically compared with residual cancerous lesions.

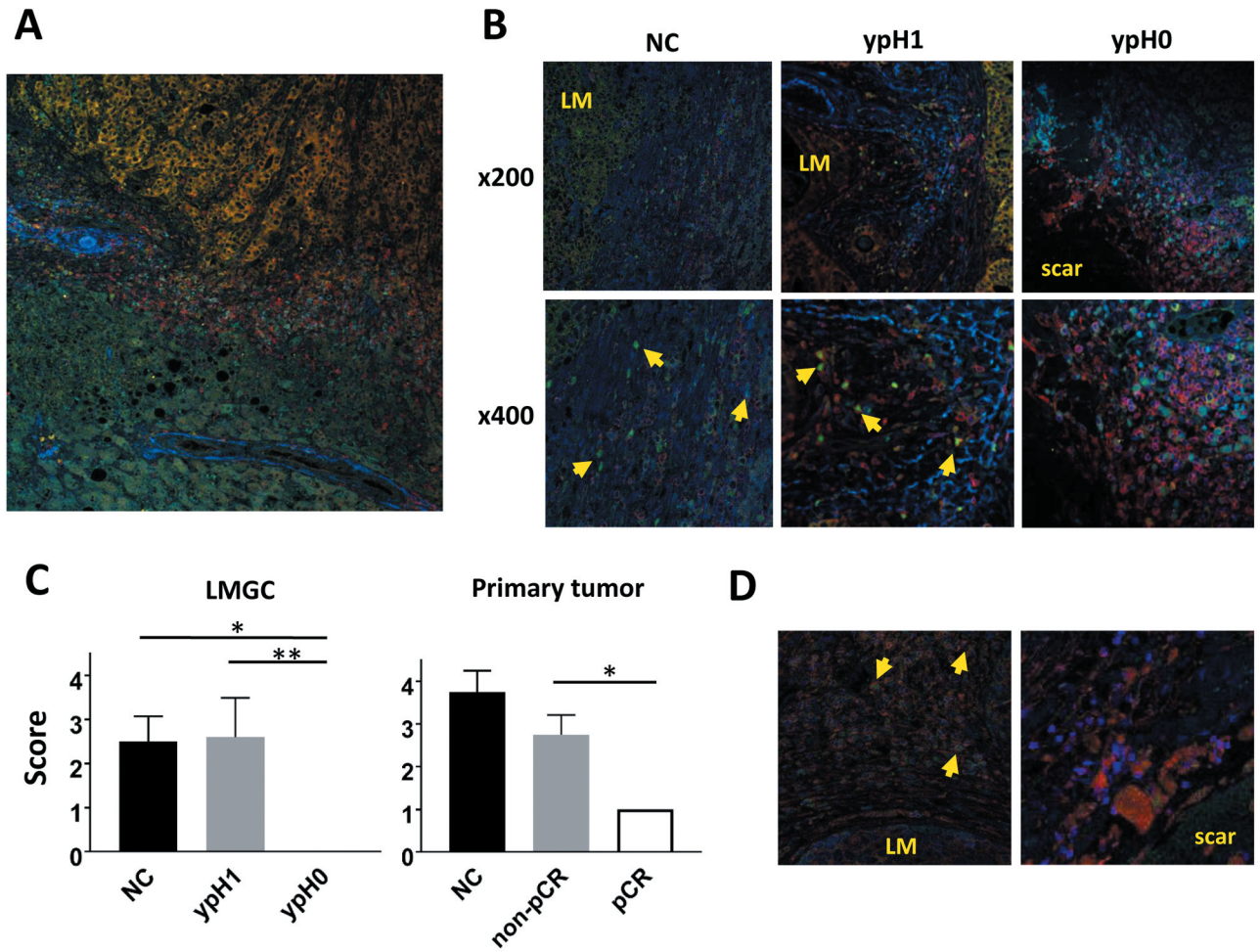


Figure 2. *Markedly fewer CD3⁺ Foxp3⁺ cells were detected in tumor scar tissue than in residual liver metastases from gastric cancer. (A) CD3, Foxp3, and the nucleus were stained with anti-CD3 and anti-Foxp3 antibody and DAPI, and visualized by immunohistochemistry of the liver metastases from patient #8 (100×) (CD3: red, Foxp3: green, DAPI: blue). (B) Immunohistochemical examination of CD3⁺ Foxp3⁺ cells in three groups [NC, hepatectomy without chemotherapy (n=4); ypH1, histological residual cancer after chemotherapy (n=5); ypH0, tumor scar after chemotherapy (n=4)]. (C) Percentages of CD3⁺ Foxp3⁺ cells in CD3⁺ cells in the liver metastases and in the primary tumor [NC, hepatectomy without chemotherapy (n=4); non-pCR, histological residual cancer after chemotherapy (n=8); pCR, tumor scar after chemotherapy (n=2)] (*p<0.05, **p<0.01). (D) Immunohistochemical examination of CD3⁺ Foxp3⁺ cells in both residual tumor and tumor scar tissue in patient #10 (400×).*

## Discussion

The relationship between the efficacy of chemotherapy and Ti-Tregs in gastric cancer and liver metastases has been unclear. In this study, we analyzed the number of tumor-infiltrating $CD3^+$ $Foxp3^+$ cells in patients who underwent R0 surgery after chemotherapy. In approximately half of the cases, LMGC disappeared histologically despite radiological evidence of residual disease. In the tumor scar of lesions that disappeared pathologically, $CD3^+$ $Foxp3^+$ cells were almost completely absent, in contrast to residual LMGC. Our results indicate that an understanding of the role of Ti-Tregs might improve the clinical outcome of patients with LMGC.

A review showed that the 5-year survival rate was 20% in patients who underwent R0 resection for LMGC, and the prognosis was poor despite multidisciplinary treatment (2). Systemic chemotherapy was recently recommended as the initial treatment in patients with multiple liver metastases (15). Moreover, Arigami *et al.* reported that response to chemotherapy was a prognostic factor in patients who underwent conversion surgery for LMGC (16). In our study, some patients showed a pathological complete response, despite having radiological and macroscopic evidence of residual LMGC. This finding suggests that a new biomarker for disappearance of cancer tissue is strongly needed to prevent unnecessary hepatectomy.

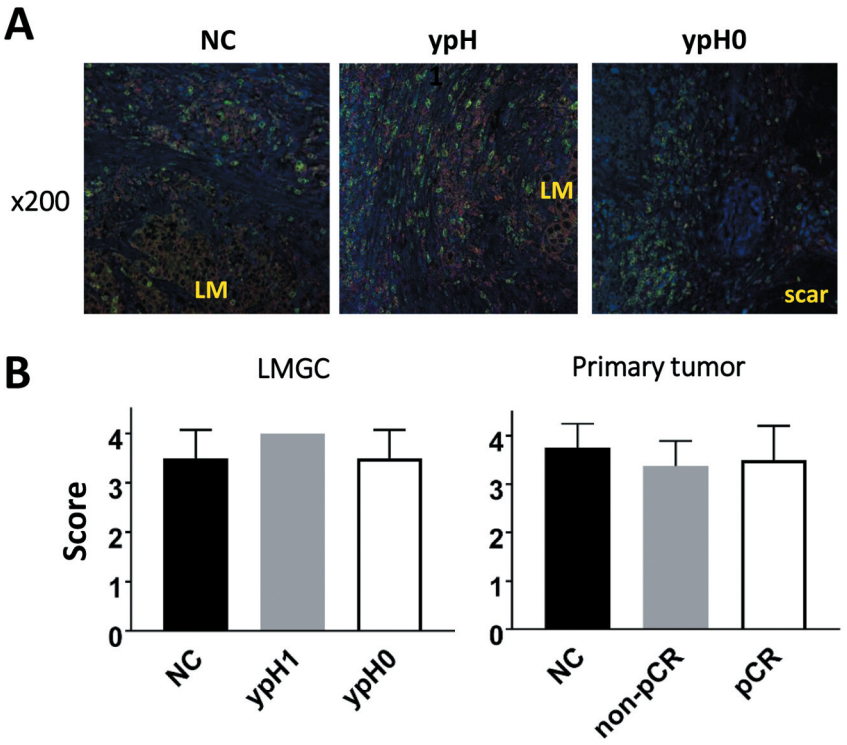


Figure 3. *Lack of correlation between the frequency of tumor-infiltrating CD8+ cells and pathological efficacy of chemotherapy. (A) CD8 and CD3 status was determined by immunohistochemistry in the three groups (CD8: green, CD3: red, DAPI: blue). (B) Percentages of CD8+ cells in CD8+ cells and/or CD3+ cells in liver metastases from gastric cancer and in the primary tumor.*

Tregs inhibit proliferation of effector T-cells because of their capacity for self-proliferation and production of interleukin-10 (7, 17). Tregs play an important role in *Helicobacter pylori*-related gastric cancer (18). In human blood and ascites samples, both the percentage of $CD4^+$ $CD25^+$ T-cells and the number of interleukin-10-producing cells correlated with the stage of gastric cancer (19). In breast cancer, pathological complete remission after neoadjuvant chemotherapy correlated with the number of Ti-Tregs (10). Furthermore, a relationship was found between the number of Ti-Tregs and pathological complete remission after neoadjuvant chemoradiotherapy for esophageal cancer (11). In our present study, the number of Ti-Tregs was markedly decreased at the invasive front of cancer tissue in the liver and the primary tumor scar compared with residual cancer lesions. The dramatic reduction in Ti-Tregs in the liver tumor scar tissue is consistent with Tregs being almost completely absent in the normal liver tissue in contrast to the gastric mucosa. The percentage of $CD8^+$ cells including $CD8^+$ T-cells showed almost no difference between residual cancer lesions and tumor scar tissue. This finding confirms that histological evidence of chemotherapeutic efficacy correlates specifically with the number of Ti-Tregs. This is the first report on Ti-Tregs in LMGC. These findings suggest that Ti-Tregs are an important biomarker of the efficacy of systemic chemotherapy for LMGC. Positron emission tomography has been used to detect immune cells with labeled extracellular proteins, such as CD34 and CD20 (20). In the future, this technology might be useful analyzing Ti-Tregs as a biomarker of pathological disappearance of tumors.

The mechanism for the relationship between the marked reduction in the number of Ti-Tregs and the disappearance of cancer remains unclear. It has been hypothesized that down-regulation of tumor-produced TGF-β1 and CCL17/22 derived from macrophages and dendritic cells inhibits induction of Ti-Tregs and migration of $CCR4^+$ Tregs to the tumor site (21, 22). There has been a focus on the efficacy of anti-Treg therapy because Ti-Tregs promote tumor progression. Placebo-controlled studies in unresectable gastric cancer have found that the anti-PD-1 monoclonal antibodies nivolumab and pembrolizumab improved both relapse-free survival and 5-year survival rates (23, 24). Microsatellite instability, Epstein-Barr virus positivity and Glasgow prognostic score were reported as the biomarker of the anti-PD-1 monoclonal antibody (25, 26). A clinical trial of combination therapy with nivolumab and ipilimumab, anti-CTLA-4 antibody, for unresectable gastric cancer is ongoing (27). The anti-CCR4 antibody mogamulizumab is

known to be effective for cutaneous T-cell lymphoma (28). Clinical trial of mogamulizumab plus nivolumab for gastric and esophageal is in progress (27). Our finding that Ti-Tregs infiltrated viable cancer tissue but not fibrotic tissue suggests that these cells play a role in maintenance of tumor growth and that treatment targeted to tumor-related Tregs would be effective.

A limitation of this study is that the presence of liver metastases before chemotherapy was not confirmed by histological examination. We did not perform needle biopsy for liver metastases to prevent dissemination of cancer tissues. However, the diagnosis of liver metastases by both contrast-enhanced ultrasonography improves sensitivity and specificity to 80-98% and 83-98%, respectively, compared with ultrasonography alone (29). Furthermore, the use of both contrast-enhanced ultrasonography and contrast-enhanced computed tomography allows more precise diagnosis of liver metastases (30). Therefore, histological examination before chemotherapy was not necessary for this research.

Marked reduction in Ti-Tregs was well correlated with the pathological complete remission of LMGC after chemotherapy. Ti-Tregs may be a biomarker that can predict the effects of chemotherapy when used in combination with radiological findings, which do not always reflect therapeutic effects.

## Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

## Authors' Contributions

YN acquired radiological, surgical, and experimental data and drafted the manuscript. YN and SH designed experiments. SH, YH, TN, TK, YK, YI, and HS contributed to final revision of the manuscript. HS supervised the study. All Authors read and approved the final manuscript.

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