Cetuximab strongly enhances immune cell infiltration into liver metastatic sites in colorectal cancer

Yuka Inoue,1 Shoichi Hazama,1,2 Nobuaki Suzuki,1 Yukio Tokumitsu,1,2 Shinsuke Kanekio,1 Shinobu Tomochika,1 Ryuichi Tsunedomi,1 Yoshihiro Tokuhisa,1 Michihisa Iida,1 Kazuhiko Sakamoto,1 Shigeru Takeda,1 Tomio Ueno,1 Shigefumi Yoshino1,3 and Hiroaki Nagano1

1Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi; 2Department of Translational Research and Developmental Therapeutics against Cancer, Yamaguchi University School of Medicine, Ube, Yamaguchi; 3Oncology Center, Yamaguchi University Hospital, Ube, Yamaguchi, Japan

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Correspondence
Hiroaki Nagano, Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube city, Yamaguchi 755-8505, Japan. Tel: +81-836-22-2264; Fax: +81-836-22-2263; E-mail: hnagano@yamaguchi-u.ac.jp

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Cetuximab has activity against colorectal cancers. Recent studies demonstrated that cetuximab induces antibody-dependent cell-mediated cytotoxicity via immune cells, and a new immune-related mechanism of inducing immunogenic cell death. This study aimed to evaluate the immune responses induced by cetuximab in tumor microenvironments at liver metastasis sites of metastatic colorectal cancer patients. We assessed immune cell infiltration in the liver metastatic sites of 53 colorectal cancer patients. These patients were divided into three groups according to the treatment before operation: chemotherapy with cetuximab, chemotherapy without cetuximab, and no chemotherapy. The inflammatory cells in the liver metastatic sites were assessed by hematoxylin–eosin staining, focusing on the invasive margin. The overall inflammatory reaction and number of lymphoid cells were assessed with a four-point scoring system. We then assessed immune cell infiltration (CD3, CD8 and CD56) in 15 liver metastatic sites. Hematoxylin–eosin staining demonstrated more inflammatory cells in the chemotherapy with cetuximab group than in the other groups (P < 0.001). Of note, inflammatory cells were found in intratumoral areas, and the destruction of cancer cell foci was observed in the chemotherapy with cetuximab group. Moreover, a higher infiltration of CD3+ (P = 0.003), CD8+ (P = 0.003) and CD56+ (P = 0.001) cells was observed in the chemotherapy with cetuximab group than in the other groups. These results suggest that cetuximab might have an immune-enhancing effect. As such, the immune-related mechanism of action of cetuximab may enhance the efficacy of combination therapy, such as chemotherapy and immunotherapy using therapeutic peptides.
characters of the T cell repertoire (TCR) before and after treatment in tumor and blood lymphocytes; the study revealed that tumor and blood lymphocytes shared the same few TCRs. Hence, we concluded that some agent that can recruit lymphocytes from the blood to the tumor site must be required for immunotherapy to be effective. In this study, we used resected liver metastasis specimens from mCRC patients, who were treated or not treated with chemotherapy in combination with or without cetuximab, to evaluate the influence of cetuximab on lymphocyte infiltration into the tumor microenvironment, and the potential value of cetuximab-induced ADCC.

Materials and Methods

Patients and tissue samples. The study materials consisted of a consecutive series of 53 specimens from liver metastatic sites of mCRC patients who underwent hepatectomy at the Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine (Yamaguchi, Japan), from 2002 to 2016. Our treatment strategy for colorectal liver metastases (CLM) is as follows. Patients with initially resectable CLM received neoadjuvant chemotherapy and were converted from unresectable to resectable. Chemotherapy regimens were chosen according to the IRB-approved clinical studies, focusing on the IM. IM was defined as the interface between the host stroma and the invading edge area of the tumor. For estimation of the inflammatory cell reaction, areas with the deepest invasion were selected. The overall inflammatory reaction and the number of lymphoid cells, and neutrophilic and eosinophilic granulocytes were assessed by using a four-point scoring system. Recently, our first-line regimens have been oxaliplatin-based chemotherapy for KRAS wild-type metastatic colorectal cancer based on our previous clinical study. The cases with a complete response (CR) to chemotherapy before operation were excluded from this study.

Sections were cut at a thickness of 4 μm from paraffin-embedded tissue blocks, mounted on silanized slides, and subsequently dewaxed and rehydrated using xylene and graded alcohol washes. We assessed the inflammatory cell reaction by examining hematoxylin–eosin (HE)-stained specimens for all cases. Then, we performed an additional assessment of the infiltrated immune cells by immunohistochemical (IHC) staining for 15 of the 53 patients.

Inflammatory cell reaction scoring by HE staining. Inflammatory cell reaction was assessed by examining HE-stained specimens, focusing on the IM. IM was defined as the interface between the host stroma and the invading edge area of the tumor. For estimation of the inflammatory cell reaction, areas with the deepest invasion were selected. The overall inflammatory reaction and the number of lymphoid cells, and neutrophilic and eosinophilic granulocytes were assessed by using a four-point scoring system. Figure 1 shows the spectrum of the inflammatory cell reactions at the IM. A score of 0 was given when there was no increase in inflammatory cells (Fig. 1a). A score of 1 denoted a mild and patchy increase in inflammatory cells at the IM with no destruction of invading cancer cell islets by the inflammatory cells (Fig. 1b). A score of 2 was given when inflammatory cells formed a band-like infiltrate at the IM (Fig. 1c). A score of 3 denoted a very prominent inflammatory reaction with the formation of a cup-like zone at the IM, and the destruction of cancer cell islets was frequent and invariably present (Fig. 1d).

Inflammatory cell reaction scoring by HE staining. Inflammatory cell reaction was assessed by examining HE-stained specimens, focusing on the IM. IM was defined as the interface between the host stroma and the invading edge area of the tumor. For estimation of the inflammatory cell reaction, areas with the deepest invasion were selected. The overall inflammatory reaction and the number of lymphoid cells, and neutrophilic and eosinophilic granulocytes were assessed by using a four-point scoring system. Recently, our first-line regimens have been oxaliplatin-based chemotherapy for KRAS wild-type metastatic colorectal cancer based on our previous clinical study. The cases with a complete response (CR) to chemotherapy before operation were excluded from this study.

Fig. 1. Spectrum of inflammatory cell reactions at the invasive margin (IM). The generalized inflammatory cell infiltrate at the margin was graded according to a 4-point score. A score of 0 was given when there was no increase in inflammatory cells (a). A score of 1 was given when there was a mild and patchy increase in inflammatory cells (b). A score of 2 was given when inflammatory cells formed a band-like infiltrate (c). A score of 3 was given when there was a very prominent inflammatory reaction that formed a cup-like zone (d). Bar = 200 μm.
CD3 (undiluted; clone 2GV6; Ventana, Mannheim, Germany), CD8 (1:100 dilution; clone 4B11; Novocastra, Newcastle, UK) and CD56 (1:50 dilution; clone 1B6; Novocastra) were applied as the primary antibodies at room temperature for 16, 32, and 32 min, respectively.

After rinsing with phosphate-buffered saline, slides were treated with biotin-conjugated IgG in blocking solution for 8 min at room temperature. Slides were rinsed again and then incubated with streptavidin-conjugated horseradish peroxidase in blocking solution for 8 min at room temperature. Protein signals were developed by DAB and hydrogen peroxide for 8 min at 37°C. Slides were finally incubated with copper for 4 min to enhance the signal intensity.

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The IHC staining for CD3 and CD8 at the IM was graded to be either low density infiltrate or high density infiltrate (Fig. 2). Low density was defined as no/weak or moderate infiltration at the IM, and high density was defined as strong and massive infiltration at the IM. NK cell reaction at the IM was detected by IHC staining for CD56; the staining was graded as either absent (a) or present (b). Bar = 200 μm.

For CD56-positive cells, two distinct immunologic patterns were clearly observed: either undetectable CD56 staining or positive CD56 staining.

**Statistical analysis.** The SPSS program (version 20; SPSS, Chicago, IL, USA) was used for statistical analysis. Pearson’s χ² test was used unless otherwise stated. All P-values were two-sided and were considered to be statistically significant when <0.05.

**Results**

**Patient characteristics.** The patient characteristics are showed in Table 1. Patients were divided into three groups according to the treatment before operation as follows: 24 patients received no treatment before operation (no chemotherapy group); 16 patients received chemotherapy without cetuximab (chemotherapy without cetuximab group); and 13 patients received chemotherapy with cetuximab (chemotherapy with cetuximab group). The chemotherapy regimen and associated objective response (OR) are shown in Table 1. In the chemotherapy without cetuximab group, the best clinical
response was partial response (PR) in five patients, stable disease (SD) in five patients and progressive disease (PD) in six patients. The PD cases received the mFOLF / FOLFIRI regimen in two cases, FOLFIRI regimen in one case, 5'-DFUR + irinotecan regimen in two cases and 5-Fu + LV regimen in one case. In the chemotherapy with cetuximab group, the best clinical response was PR in 11 patients and SD in the other groups. Of note, inflammatory cells were found in intratumoral areas, and the destruction of cancer cell foci was observed in the chemotherapy with cetuximab group. The two cases that received a score of 1 in the chemotherapy with cetuximab group were both cases with SD. We have summarized the inflammatory cell infiltration focused on PR cases (Table S1). The immune cell infiltration in the chemotherapy with cetuximab group was stronger than that in the chemotherapy without cetuximab group (P = 0.005).

Assessment of inflammatory cell reaction by immunohistochemistry. Table 3 shows the relationships between the treatment modalities and the expression of surface markers (CD3, CD8 and CD56). Comparison of the three groups revealed that CD3 and CD8 were more highly expressed in the chemotherapy with cetuximab group than in the other groups (P = 0.003 for both). In addition, CD56 was more highly expressed in the chemotherapy with cetuximab group than in the other groups (P = 0.001).

Discussion
The major mechanism of action of cetuximab was thought to be mediated by the blockade of the EGFR-ligand interaction and downstream signaling.(11) One possible mechanism is ADCC mediated through the FcγR(14) and another new immune-related mechanism of action of cetuximab is the induction of immunogenic cell death in combination with chemotherapy.(45)
In general, patients with good response were associated with enriched immune cells even with chemotherapy alone. The current study demonstrated the ability of cetuximab to recruit immune cells not only to the tumor margin, but also into the tumor microenvironment surrounding the tumor cell nest of the mCRC lesion. HE staining analysis revealed significantly more inflammatory cells in the chemotherapy with cetuximab group than in the chemotherapy without cetuximab group. Furthermore, interestingly, comparing PR cases between the chemotherapy with cetuximab and chemotherapy without cetuximab groups, inflammatory cells had infiltrated into the intratumoral areas in the majority of cases in the chemotherapy with cetuximab group, in contrast to the chemotherapy without cetuximab group, in which inflammatory cells were located in peritumoral areas.

In the IHC analysis, the incidence of CD56-positive cells was also significantly higher in the chemotherapy with cetuximab group than in the other groups. As mentioned above, CD56-positive cells, mainly NK cells, may be the major effector of cetuximab-mediated ADCC, and our results provided evidence that CD56+ cells are effectors of cetuximab-mediated ADCC in mCRC patients.

Furthermore, comparisons of the three groups revealed that CD3+ and CD8+ were more highly expressed on T cells in the chemotherapy with cetuximab group than in the other groups. These results are supported by reports that cetuximab might induce immunogenic cell death in combination with chemotherapy, and cetuximab might have some sort of immune-enhancing effect, including ADCC activity, following the accumulation of CD8+ cytotoxic T cells in the tumor microenvironment.

Taken into consideration with the ability of cetuximab to induce immune cell infiltration into tumor sites, and since prior immune cell infiltration into tumor sites is required for the majority of immunochemotherapies to be effective, the immune-related mechanism of action of cetuximab may help enhance the efficacy of combination therapy, such as chemotherapy and immunotherapy using therapeutic peptide vaccination.

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Disclosure Statement
All authors have no conflict of interest to declare. All authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

Abbreviations
ADCC antibody-dependent cellular cytotoxicity
CLM colorectal liver metastasis
CR complete response
CRC colorectal cancer
DAB diaminobenzidine
EGFR epidermal growth factor receptor
FcγR Fc gamma receptors
HRP horseradish peroxidase
IgG immunoglobulin G1
IHC immunohistochemistry
IM invasive margin
mCRC metastatic colorectal cancer
NK natural killer
PD progressive disease
PD-1 programmed death 1
PR partial response
SD stable disease

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Table S1. Assessment of peritumoral infiltration of the inflammatory cells depend on the treatment before resection for only PR cases.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Assessment of peritumoral infiltration of the inflammatory cells depend on the treatment before resection for only PR cases.