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

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Key words

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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.

Colorectal cancer (CRC) is among the leading causes of mortality and morbidity throughout the world, thus representing a major public health concern. It is the third most common cancer worldwide, and the fourth most common cause of oncological death.[1,2] In Japan, approximately 125 000 new cases of CRC are diagnosed each year.

Nowadays, several drugs can be used to treat CRC.[3–10] Cetuximab, a chimeric immunoglobulin G1 (IgG1) monoclonal antibody targeting the extracellular domain of epidermal growth factor receptor (EGFR),[11] is one of the drugs that have shown efficacy in patients with metastatic CRC (mCRC).[10,12] The mechanism of cetuximab has largely been attributed to the direct anti-proliferative and pro-apoptotic effects of the antibody; moreover, antibody-dependent cellular cytotoxicity (ADCC), which is mediated through the Fc gamma receptors (FcγR)[13] on immune cells, such as natural killer (NK) cells and macrophages, also plays an important role in the antitumor effect of the IgG1 antibody.[14] Marechal *et al.* have already reported that CD56-positive cells, mainly NK cells, may be the major effector of ADCC-related cetuximab activity.[15–17]

The role of the number or density of immune cells is increasingly being recognized in determining cancer-specific survivals in CRC.[18] The evidence was strongest for a generalized lymphocytic/inflammatory cell infiltrate at the invasive margin (IM),[18–20] and most works have focused on T lymphocytes and their subsets (CD3+, CD4+, CD8+, CD45RO+ and FOXP3+) and macrophages (CD68).[18,21–23] A prognostic score, "Immunoscore", which takes into account the distribution of the density of both CD3+ lymphocytes and CD8+ cytotoxic T cells in the tumor core and IM, was reported, and it could outperform TNM staging.[24,25] The characterization of immune infiltrates could also provide information for predicting the disease outcome in patients treated with checkpoint-blockade strategies,[26] and is also considered to be important for predicting the therapeutic response to immunochemotherapy.[26–29]

We have reported a phase I study of a peptide vaccine with chemotherapy against mCRC,[30] and analyzed the

characteristics 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.[31] 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 in principle.[32] Patients, who might be undergone simple hepatectomy such as single partial hepatectomy or lateral segmentectomy, received surgery without neoadjuvant chemotherapy. All patients with borderline resectable or unresectable CLM received chemotherapy, and then the resectability of CLM was considered. In this study, only one of the 53 patients was converted from unresectable to resectable. Chemotherapy regimens were chosen according to the IRB-approved clinical studies, which have been ongoing during the study period.[33–40] Recently, our first-line regimens have been oxaliplatin-based regimens such as mFOLFOX (bolus/infusional fluorouracil and leucovorin with oxaliplatin) or XELOX (capecitabine and oxaliplatin) in combination with anti-VEGF antibody or anti-EGFR antibody.[40] In particular, we selected cetuximab as the anti-EGFR antibody rather than panitumumab, in combination with standard chemotherapy for KRAS wild-type metastatic colorectal cancer based on our previous clinical study.[41]

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.[42–44] 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).

Immunohistochemistry. We assessed immune cell infiltration (CD3, CD8 and CD56) in 15 of the 53 specimens from liver metastatic sites of the CRC patients.

Immunohistochemical staining was performed for CD3, CD8 and CD56 on an automatic staining machine (Bench Mark XT; Ventana Medical Systems, Tucson, AZ, USA) using the iVIEW 3,3-diaminobenzidine (DAB) detection kit (Ventana Medical Systems). After tissue sections (4 μm) on slides were deparaffinized and hydrated, they were heated to 100°C for 60 min to induce antigen retrieval using Ventana Cell Conditioner 1 (Ventana Medical Systems). Slides were treated with inhibitor at 37°C for 4 min to inactivate endogenous peroxidase activity. Rabbit monoclonal antibodies recognizing human

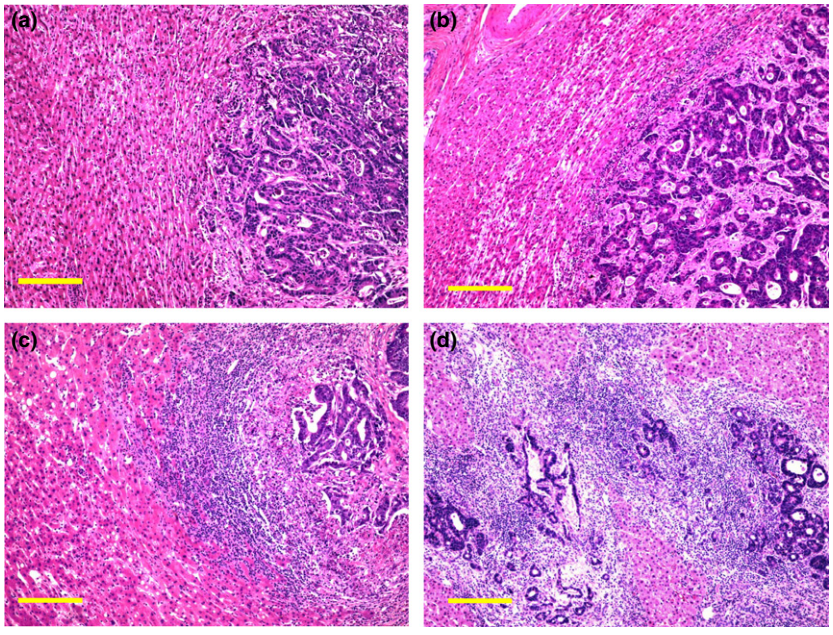

Fig. 1. Spectrum of inflammatory cell reactions at the invasive margin (IM). The generalized inflammatory cell infiltrate at the margin was graded according to a 4-point score. A score of 0 was given when there was no increase in inflammatory cells (a). A score of 1 was given when there was a mild and 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.

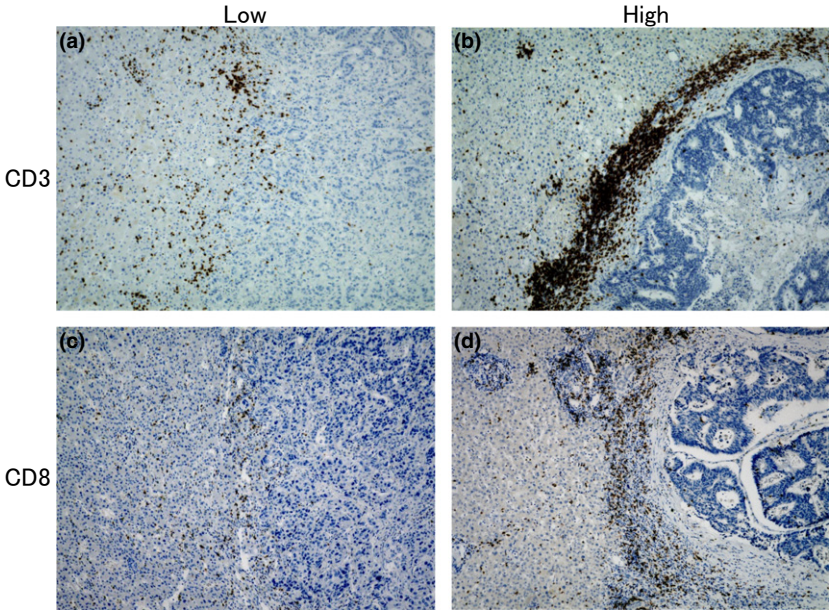

Fig. 2. Invasive margin of liver metastases with different infiltrate densities. The immunohistochemical staining for CD3 and CD8 at the invasive margin was graded as either low density infiltrate (a, c) or high density infiltrate (b, d). Bar = 200 μm.

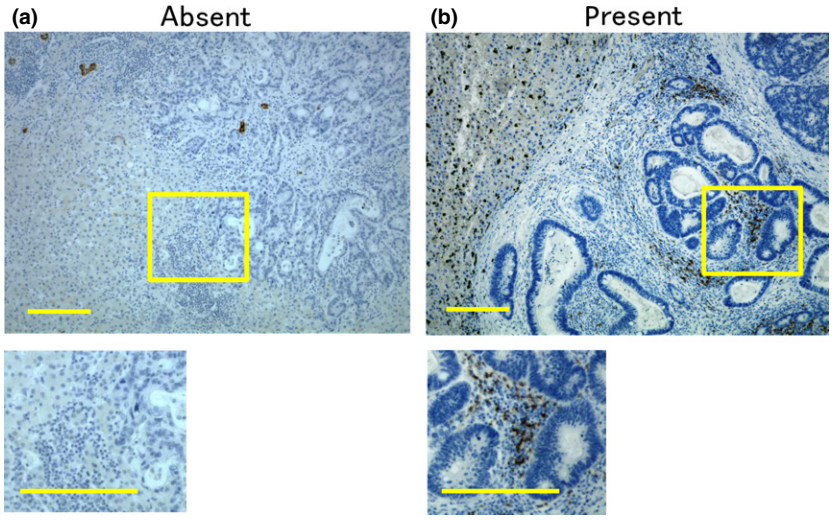

Fig. 3. Natural killer (NK) cell reaction at the invasive margin (IM) was detected by immunohistochemical staining for CD56. The NK cell reaction was graded as either absent (a) or present (b). Bar = 200 μm.

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.

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 or present (Fig. 3). 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 χ^2 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

Table 1. Patient characteristics

Characteristic	Without CT *n* = 24	CT without Cmab *n* = 16	CT with Cmab *n* = 13
Age, average (range)	64.2 (45–84)	62.4 (48–78)	64.4 (39–78)
Sex			
Male	10	8	10
Female	14	8	3
Chemotherapy regimen (OR)			
mFOLFOX	–	3 (2)	4 (3)
mFOLFOX + bevacizumab	–	5 (2)	–
XELOX	–	0	8 (6)
XELOX + bevacizumab	–	1 (0)	–
FOLFIRI	–	2 (1)	1 (1)
5′–DFUR + Irinotecan	–	3 (0)	0
5-Fu + LV	–	2 (0)	0
RECIST			
CR	–	–	–
PR	–	5	11
SD	–	5	2
PD	–	6	0

Cmab, cetuximab; CR, complete response; CT, chemotherapy; 5′-DFUR, 5′-deoxy-5-fluorouridine; FOLFIRI, bolus/infusional fluorouracil and leucovorin with irinotecan; LV, leucovorin; mFOLFOX, bolus/infusional fluorouracil and leucovorin with oxaliplatin; OR, objective response (CR + PR) by RECIST version 1.1; PD, progressive disease; PR, partial response; RECIST, response evaluation criteria in solid tumors; SD, stable disease; XELOX, capecitabine and oxaliplatin.

Table 2. Assessment of peritumoral infiltration of the inflammatory cells depend on the treatment before resection

Treatment before resection	4-Point score				*P*-value
	0	1	2	3	
Without CT	5	9	9	1	<0.001
CT without Cmab	6	5	5	0	
CT with Cmab	0	2	1	10	

Cmab, cetuximab; CT, chemotherapy.

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 mFOLFOX + bevacizumab 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 two patients. The reason for the marked differences in OR between the chemotherapy with cetuximab and chemotherapy without cetuximab groups is that the chemotherapy regimen for colorectal cancer was developed during this study period. Three of six PD cases in the chemotherapy without cetuximab group were enrolled at the beginning of this study period and received the 5-Fu + LV or 5′-DFUR + irinotecan regimen.

We assessed the immune cell infiltrates (CD3, CD8 and CD56) in 15 of the 53 specimens from liver metastatic sites of the CRC patients. We assessed seven cases from the chemotherapy with cetuximab group that were operated on before 2013; of these, six cases were treated with an oxaliplatin-containing regimen, and one was treated with an irinotecan-containing regimen. As a control, we assessed four cases from the chemotherapy without cetuximab group that were operated on before 2013; all of these cases received an oxaliplatin-containing regimen, and the best clinical response was a PR for all cases. In addition, four cases were selected from the no chemotherapy group at random.

Assessment of inflammatory cell reaction by HE staining. The assessments of inflammatory cell infiltration at the IM according to the treatment before resection are shown in Table 2. In the HE staining analysis, there was significantly more infiltration of inflammatory cells in the chemotherapy with cetuximab group than in the other groups ($P < 0.001$). Almost all of the cases in the chemotherapy with cetuximab group had a score of 3, whereas little infiltration of inflammatory cells was seen 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]

Table 3. Assessment of immune cells infiltrate by IHC depend on the treatment before resection

Treatment before resection	CD3			CD8			CD56		
	Low	High	*P*-value	Low	High	*P*-value	–	+	*P*-value
Without CT	4	0	0.003	4	0	0.003	4	0	0.001
CT without Cmab	4	0		4	0		3	1	
CT with Cmab	1	6		1	6		0	7	

Cmab, cetuximab; CT, chemotherapy; IHC, immunohistochemistry.

In general, patients with good response were associated with enriched immune cells even with chemotherapy alone.[27–29] 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,[14,15] 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,[45] 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
IgG1	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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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.