

Association between serum ligands and the skin toxicity of anti-epidermal growth factor receptor antibody in metastatic colorectal cancer

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Key words

Colorectal cancer, EGFR, KRAS, ligands, skin toxicity

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Funding Information

The Princess Takamatsu Cancer Research Fund.

Received January 11, 2015; Revised February 16, 2015;

Accepted February 18, 2015

Cancer Sci 106 (2015) 604–610

doi: 10.1111/cas.12642

Skin toxicity is a known clinical signature used to predict the prognosis of anti-epidermal growth factor receptor (EGFR) antibody treatment in metastatic colorectal cancer (mCRC). There are no biological markers to predict skin toxicity before anti-EGFR antibody treatment in mCRC patients. Between August 2008 and August 2011, pretreatment serum samples were obtained from *KRAS* wild-type (WT) patients who received anti-EGFR antibody treatment. Serum levels of ligands were measured by ELISA. A total of 103 *KRAS* WT patients were enrolled in the study. Progression-free survival and overall survival of patients with a high grade (grade 2–3) of skin toxicity were significantly longer than those with a low grade (grade 0–1) of skin toxicity (median progression-free survival, 6.4 months vs 2.4 months, $P < 0.001$; median overall survival, 14.6 months vs 7.1 months, $P = 0.006$). There were significant differences in distribution of serum levels of epiregulin (EREG), amphiregulin (AREG), and hepatocyte growth factor (HGF) between groups of low/high grade of skin toxicity ($P < 0.048$, $P < 0.012$, $P < 0.012$, respectively). In addition, serum levels of HGF, EREG, and AREG were inversely proportional to grades of skin toxicity as determined by the Cochran–Armitage test ($P = 0.019$, $P = 0.047$, $P = 0.021$, respectively). Our study indicated that serum levels such as HGF, EREG, and AREG may be significant markers to predict the grade of skin toxicity and the prognosis of anti-EGFR antibody treatment, which contribute to improvement of the management of skin toxicity and survival time in mCRC patients.

Colorectal cancer is the third most common cancer and the third leading cause of cancer-related deaths in Japan. Survival time for mCRC patients has improved because of the new molecular target drugs that have been developed over the last decade. Anti-EGFR mAb is one of the active molecular target drugs used in chemotherapy-resistant mCRC patients.^(1–3) Recently, gene mutations of minor *KRAS*, *NRAS*, and *BRAF* mutations were recognized as predictive and prognostic factors of anti-EGFR antibody treatment in mCRC.^(4–6)

Skin toxicity is well known as a clinical signature of the response and prognosis of EGFR-target therapy in solid tumors.^(7,8) Suppression of the EGFR signal pathway injures keratinocytes by inducing growth arrest and apoptosis, decreasing cell migration, and increasing cell attachment, cell differentiation, and stimulating inflammatory chemokine expression.⁽⁹⁾ Some previous articles have reported on the expression and localization of EGFR and EGFR ligands in human skin, and the phenotypes of knockout and transgenic mice developed to analyze the *in vivo* function of the EGFR/ligand system in the skin.⁽¹⁰⁾

Ligands of the ErbB family in humans consist of EGF, TGF- α , heparin binding-EGF, betacellulin, AREG, EREG, epi-

gen, and NRG. Hepatocyte growth factor/scatter factor and IGF-1 are mesenchymal cytokines with a number of biological activities, including mitogenic, motogenic, and/or morphogenic properties in epithelial tissues.⁽¹¹⁾ Upregulation of the HGF/MET and the IGF-1/IGF-1 receptor pathways have been suggested as potential mechanisms of signal escape in colorectal tumors after treatment with EGFR inhibitors.^(12–14) Recently, we reported that serum levels of HGF and EREG are associated with the prognosis of anti-EGFR antibody treatment in *KRAS* WT mCRC patients.⁽¹⁵⁾

Severe skin toxicity caused by anti-EGFR antibody treatment reduces compliance and the patient's QOL. In the present study, we evaluated the association between serum levels of ligands and grade of skin toxicities due to anti-EGFR antibodies to discover the predictive markers of skin toxicity in *KRAS* WT mCRC patients.

Materials and Methods

Patients and sample collection. Between August 2008 and August 2011, specimens were collected by endoscopic biopsy or surgical resection from 337 patients with advanced CRC

and screened for the genomic status of *KRAS* codons 12 and 13 at the Gastrointestinal Oncology Division, National Cancer Center Hospital (Tokyo, Japan). Among these patients, we selected the mCRC patients who underwent anti-EGFR antibody treatment and whose tumors were *KRAS* WT (codon 12 and 13). Blood samples in our study were obtained from residual blood samples of previous laboratory tests. Separated serum was stocked at -20°C at the Biobank of clinical laboratories at the National Cancer Center Hospital until use. We selected serum samples that were taken within 2 weeks before the initiation of treatment with anti-EGFR antibodies. We enrolled the *KRAS* WT patients who met the inclusion criteria as previously described.⁽¹⁵⁾ Patients continued to receive chemotherapy until disease progression or intolerable toxicity from chemotherapy intervention. The response of treatment was evaluated by contrast-enhanced CT every 2–3 months. Informed consent from Biobank for the use of clinical materials was obtained, and this study was undertaken after approval by the institutional review board.

Treatment and evaluation of skin toxicity. All patients received anti-EGFR antibodies as combined chemotherapy or as a monotherapy. Cetuximab was given i.v. at 400 mg/m^2 on the first day, followed by 250 mg/m^2 (i.v.) weekly. Panitumumab was given at 6 mg/kg i.v. every 2 weeks. Dose reduction or drug withdrawal was carried out appropriately at the discretion of each patient's doctors. Grades of skin toxicity were evaluated using Common Terminology Criteria for Adverse Events version 4.0. The description of grades of skin toxicity in this study was defined as the worst grades of adverse events during the anti-EGFR antibody treatment. In this study, we defined "total skin toxicity" due to anti-EGFR antibody treatment as rash, acneiform eruptions, dry skin, and paronychia. Among skin toxicities caused by anti-EGFR antibody treatment, we selected acneiform eruption as acute toxicity and paronychia as late toxicity.

Enzyme-linked immunosorbent assay. We selected the ligands EGF, TGF- α , AREG, EREG, NRG, HGF, and IGF-1, which were previously reported to be associated with the activation and cross-talk of the EGFR downstream signaling pathway in solid tumors. We used ELISA kits to measure serum levels of ligands as follow: Human HGF Quantikine ELISA Kit (DHG00; R&D Systems, Minneapolis, MN, USA), Human Epiregulin ELISA kit (CSB-EL007779HU; CUSABIO, Wuhan, China), Human Amphiregulin ELISA kit (E90006Hu; USCN Life Science, Wuhan, China), Human EGF Quantikine ELISA kit (DEG00; R&D Systems), Human TGF- α Quantikine ELISA kit (DTGA00; R&D Systems), Human Neureglin-1 ELISA kit (CSB-E17153 h; CUSABIO), and Human IGF-1 Quantikine ELISA kit (DG00; R&D Systems). Protocols of ELISA for these ligands are summarized in Table S1.

Direct sequencing of *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*. DNA samples were extracted from formalin-fixed paraffin-embedded tumor tissue sections. Tumor cell-rich areas in the H/E section were marked under a microscope, and tissue was scraped from the corresponding area of another deparaffinized unstained section. DNA from the scraped-off tissue sample was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen KK, Tokyo, Japan). Exons 2 (codon 12, 13), 3 (codon 61), and 4 (codon 146) of the *KRAS* gene, exon 15 (codon 600) of the *BRAF* gene, exons 9 (codon 542, 545) and 20 (codon 1047) of the *PIK3CA* gene, and exons 2 (codon 12, 13) and 3 (codon 61) of the *NRAS* gene were amplified by PCR using the GeneAmp PCR System 9700 Thermal Cycler (Life Technologies Japan [Applied Biosystems], Tokyo, Japan). The PCR products were visualized

using agarose gel electrophoresis with ethidium bromide staining and directly sequenced using an ABI 3130x/Genetic Analyzer (Life Technologies Japan [Applied Biosystems], Tokyo, Japan) according to the manufacturer's instructions.

Assessment and statistical analysis. Progression-free survival was defined as the interval from initiation of anti-EGFR antibody treatment to the occurrence of disease progression or death without evidence of progression. Overall survival was defined as the interval from initiation of anti-EGFR antibody treatment to death or last follow-up. Survival curves for OS and PFS were estimated using the Kaplan–Meier method, and differences were evaluated with the log–rank test. Hazard ratios of skin toxicity (high grade/low grade) adjusted for age (cut-off, median); gender (male/female); performance status (0–1/2); primary site (colon/rectum); histological type (differentiated/undifferentiated); number of metastatic sites (0–1/ ≥ 2); and chemotherapy regimen (combination/monotherapy) were estimated using a Cox proportional hazards model.

We tested for monotonic trends in serum levels of each ligand over the grades of skin toxicity with the Cochran–Armitage trend test. We divided serum levels of ligands into four equal-sized groups and the grade of skin toxicity was divided into two groups, such as high grade and low grade (grade 3 vs grade 0–2, grade 2–3 vs grade 0–1, and grade 1–3 vs grade 0) for this trend test.

Differences in the distribution of two variables were evaluated using Fisher's exact test or the χ^2 -test, as appropriate. Differences in distribution of more than two variables were evaluated by the Kruskal–Wallis test. All tests were two-sided and a *P*-value <0.05 was defined as statistically significant. We carried out statistical analyses of the Kaplan–Meier method and log–rank test using SPSS statistical software, version 19 (IBM, Tokyo, Japan). The Cochran–Armitage test was carried out by SAS software version 9.3 (SAS Institute, Cary, NC, USA).

Results

Gene mutations. A total of 103 *KRAS* WT patients met the selection criteria between August 2008 and August 2011 in our hospital. Patients' characteristics are summarized in Table 1. Somatic mutations of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* in 103 patients were evaluated in this study because these mutations are known to strongly affect the response and prognosis of anti-EGFR antibody treatment. A total of 20 gene mutations were detected by direct sequencing assay in this study. The frequencies of mutations in *KRAS* codon 61, *KRAS* codon 146, *BRAF* V600E, *NRAS* codon 12/13, *NRAS* codon 61, and *PIK3CA* exon 9/20 were 1.9%, 4.9%, 1.9%, 1.9%, 3.9%, and 4.9%, respectively. Prior chemotherapy and treatment after anti-EGFR antibody treatment were described in our previous reports.⁽¹⁵⁾

Skin toxicities. The frequencies of each grade of skin toxicity were 10.7% (grade 0), 29.1% (grade 1), 47.6% (grade 2), and 12.6% (grade 3). There was a significant difference in distribution of the grade of skin toxicities between acneiform eruption and paronychia ($P = 0.021$), as shown in Table S2. Comparison of patient's background between those with a low grade (0–1) of skin toxicity and those with a high grade (2–3) of skin toxicity are summarized in Table 2. There was no statistically significant difference in terms of patients' background between these two groups.

Clinical outcomes of anti-EGFR antibody treatment by grades of skin toxicity. We evaluated the prognostic role of skin toxic-

Table 1. Baseline characteristics of patients with metastatic colorectal cancer treated with anti-epidermal growth factor receptor (EGFR) antibody (n = 103)

Patient characteristic	n (%)
Median age, years (range)	62 (29–83)
Gender	
Male	65 (63.1)
Female	38 (36.9)
ECOG PS	
0–1	98 (95.1)
2	5 (4.9)
Primary site	
Colon	52 (50.5)
Rectum	51 (49.5)
Histological type of tumor	
Well, Mod.	88 (85.4)
Por, Sig.	14 (13.6)
Muc.	1 (1.0)
Resection of primary lesion	
No	17 (16.5)
Yes	86 (83.5)
No. of metastatic sites	
1	29 (28.2)
≥2	74 (71.8)
Anti-EGFR antibodies	
Cetuximab	83 (80.6)
Panitumumab	20 (19.4)
Regimen of chemotherapy	
Monotherapy	21 (20.4)
Combination	82 (79.6)
Skin toxicity: worst grade	
Grade 0	11 (10.7)
Grade 1	30 (29.1)
Grade 2	49 (47.6)
Grade 3	13 (12.6)
Grade 4	0 (0.0)
Genomic mutations	
KRAS codon 61	2 (1.9)
KRAS codon 146	5 (4.9)
BRAF V600E	2 (1.9)
NRAS codon 12, 13	2 (1.9)
NRAS codon 61	5 (4.9)
PIK3CA exon 9, 20	4 (3.9)

ECOG, Eastern Cooperative Oncology Group; Mod, moderately differentiated; Muc., mucinous adenocarcinoma; Por, poorly differentiated; PS, performance status; Sig., signet ring cell carcinoma; Well, well differentiated.

ities not only in *KRAS* WT patients but also in patients of all WTs of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA*. Survival curves estimated by the Kaplan–Meier method are summarized in Figure S1. Among *KRAS* WT patients, there was a significant difference in median PFS by grades of total skin toxicity (grade 3, 7.5 months; grade 2, 6.4 months; grade 1, 3.5 months; grade 0, 1.3 months; $P < 0.001$). When patients were divided into groups according to grade, the median PFS in the high grade group and low grade group were 6.4 months (95%CI, 4.8–8.0) and 2.4 months (95%CI, 1.6–3.3), respectively. There was a significant difference in PFS between these two groups ($P < 0.001$). In addition, the median OS of the high grade and low grade groups were 14.6 months (95%CI, 12.0–17.3) and 7.1 months (95%CI, 5.6–8.9), respectively, and there was a

Table 2. Differences in characteristics in patients with metastatic colorectal cancer treated with anti-epidermal growth factor receptor (EGFR) antibody (n = 103) between those with low-grade (grades 0–1) and high-grade (grades 2–3) skin toxicity

Characteristics	Total skin toxicity		P-value
	Grades 0–1, n (%)	Grades 2–3, n (%)	
Median age, years (range)	62.0 (38–82)	61.5 (29–83)	
Gender			
Male	27 (64.3)	38 (74.5)	0.837
Female	15 (35.7)	23 (25.5)	
ECOG PS			
0–1	41 (97.6)	57 (93.4)	0.646
2	1 (2.4)	4 (6.6)	
Primary site			
Colon	23 (54.8)	29 (47.5)	0.471
Rectum	19 (45.2)	32 (52.5)	
Histological type of tumor			
Well, Mod.	37 (88.1)	51 (83.6)	0.655
Por, Sig.	5 (11.9)	9 (14.8)	
Muc.	0 (0.0)	1 (1.6)	
Resection of primary lesion			
No	10 (23.8)	7 (11.5)	0.112
Yes	32 (76.2)	54 (88.5)	
No. of metastatic sites			
1	11 (26.2)	18 (29.5)	0.825
≥2	31 (73.8)	43 (70.5)	
Anti-EGFR antibodies			
Cetuximab	32 (76.2)	51 (83.6)	0.448
Panitumumab	10 (23.8)	10 (16.4)	

Significant difference is defined as $P < 0.05$. ECOG, Eastern Cooperative Oncology Group; Mod, moderately differentiated; Muc., mucinous adenocarcinoma; Por, poorly differentiated; PS, performance status; Sig., signet ring cell carcinoma; Well, well differentiated.

significant difference in the OS between these two groups ($P = 0.001$). Adjusted HR of skin toxicity (high grade/low grade) in terms of PFS and OS were 0.609 (95%CI, 0.482–0.770; $P < 0.001$) and 0.686 (95%CI, 0.524–0.899; $P = 0.006$), respectively.

Among all-WT patients of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA*, the median PFS in the high grade and low grade groups were 8.0 months (95%CI, 5.4–10.6) and 2.5 months (95%CI, 0.0–5.4), respectively. There was a significant difference in PFS between the two groups ($P = 0.0025$). Median OS in the high grade group and low grade group were 17.6 months (95%CI, 11.5–23.7) and 7.1 months (95%CI, 5.3–9.0), respectively. There was a significant difference in OS between the two groups ($P = 0.0028$). Adjusted HR of skin toxicity in terms of PFS and OS were 0.615 (95%CI, 0.422–0.897; $P = 0.012$) and 0.563 (95%CI, 0.341–0.931, $P = 0.026$), respectively.

We also evaluated the survival curves of PFS and OS by grades of acneiform eruption and paronychia. There were significant differences in PFS and OS between low grade and high grade skin toxicity and the results are shown on Figure S2.

Results of serum levels of ligands by grades of total skin toxicity. Results of serum levels (median, range) of ligands are summarized in Table 3. Serum samples from 103 patients were used to measure the concentrations of ligands. Medians of serum EGF, TGF- α , EREG, AREG, NRG, HGF, and IGF-1 at

Table 3. Serum levels of ligands in patients with metastatic colorectal cancer treated with anti-epidermal growth factor (EGF) receptor antibody ($n = 103$)

	EGF	TGF- α	EREG	AREG	NRG	HGF	IGF-1
Serum samples, n	103	82	103	98	102	103	102
Pretreatment serum levels	(pg/mL)	(pg/mL)	(pg/mL)	(pg/mL)	(ng/mL)	(pg/mL)	(ng/mL)
Median	128.8	5.4	1485.2	27.8	67.3	1337.1	78.8
Range	14.5–818.3	0.4–73.6	562.3–3731.5	3.0–636.1	12.4–332.8	703.7–3319.3	16.9–185.2
<i>Serum levels by grade of skin toxicity</i>							
Low grade (grades 0–1)							
Median	143.7	7.3	1701.1	29.2	66.1	1503.2	64.7
Range	18.7–818.3	0.4–25.1	596.3–3731.5	4.1–636.1	17.0–180.0	703.7–3319.3	16.9–172.5
High grade (grades 2–3)							
Median	114.6	3.7	1390.5	22.4	77.2	1256.9	79.7
Range	14.5–440.0	0.0–73.6	562.3–2590.4	3.0–138.1	12.4–332.8	823.2–2669.8	17.4–185.2

Results of serum levels of EGF, transforming growth factor- α (TGF- α), epiregulin (EREG), amphiregulin (AREG), neuregulin (NRG), hepatocyte growth factor (HGF), and insulin growth factor-1 (IGF-1) are shown. Differences in serum levels of these ligands between patients with a low grade of skin toxicity and those with a high grade of skin toxicity are also shown.

pretreatment were 128.8 pg/mL, 5.4 pg/mL, 1485.2 pg/mL, 27.8 pg/mL, 67.3 ng/mL, 1337.1 pg/mL, and 78.8 ng/mL, respectively. Distribution of serum levels of ligands between low grade and high grades of skin toxicity are shown by box plot in Figure 1. Between the two groups, there were significant differences in distribution of serum levels of EREG, AREG, and HGF ($P < 0.048$, $P < 0.012$, $P < 0.012$, respectively). Differences in serum levels of ligands between patients of high grade toxicity and those of low grade toxicity are also shown in Table 3.

Results of trend tests between serum levels of ligands and grades of skin toxicity. Association between serum levels of ligands and skin toxicity were also evaluated by the Cochran–Armitage test. Grades of total skin toxicity were divided into two groups according to the following three patterns: (i) grade 0 and grades 1–3; (ii) grades 0–1 and grades 2–3; and (iii) grade 0–2 and grade 3. Results of AREG, EREG, and HGF are summarized in Table 4. When grades of skin toxicity were divided into low grade (grades 0–1) and high grade (grades 2–3), there were significant tendencies that serum levels of

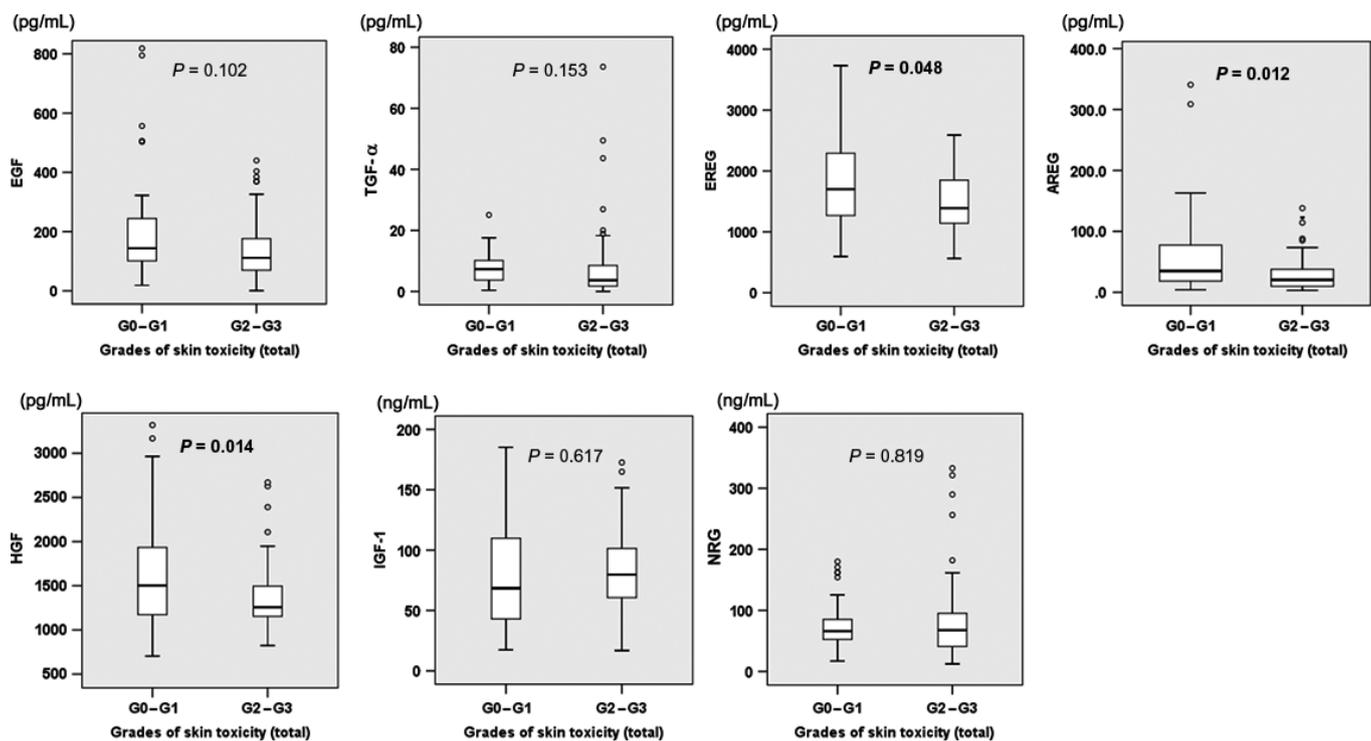


Fig. 1. Box and whisker plots illustrating the spread of data between serum ligands and the grade of total skin toxicity in patients with metastatic colorectal cancer who received anti-epidermal growth factor (EGF) receptor antibody treatment. The width of each box plot is drawn proportional to the square root of the number of observations in the groups. Skin toxicity was divided into low grade (G0–G1) and high grade (G2–G3). AREG, amphiregulin; EREG, epiregulin; HGF, hepatocyte growth factor; IGF-1, insulin growth factor-1; NRG, neuregulin; TGF- α , transforming growth factor- α .

Table 4. Results of trend test between serum levels of ligands and grades of total skin toxicity in patients with metastatic colorectal cancer treated with anti-epidermal growth factor receptor antibody ($n = 103$)

Total skin toxicity	n (%)	AREG (pg/mL)				P -value
		≤ 12.2	≤ 27.8	≤ 51.9	> 51.9	
Grade 0	10 (11.4)	0 (0.0)	4 (16.0)	3 (12.5)	3 (12.0)	0.302
Grade 1–3	88 (88.6)	24 (100)	21 (84.0)	21 (87.5)	22 (88.0)	
Grade 0–1	38 (38.8)	3 (12.5)	13 (52.0)	9 (37.5)	13 (52.0)	0.021
Grade 2–3	60 (61.2)	21 (87.5)	12 (48.0)	15 (62.5)	12 (48.0)	
Grade 0–2	86 (87.8)	18 (75.0)	24 (96.0)	21 (87.5)	23 (92.0)	0.174
Grade 3	12 (12.2)	6 (25.0)	1 (4.0)	3 (12.5)	2 (8.0)	

Total skin toxicity	n (%)	EREG (pg/mL)				P -value
		≤ 1172.5	≤ 1485.2	≤ 2097.0	> 2097.0	
Grade 0	11 (10.7)	1 (4.0)	2 (7.4)	2 (8.0)	6 (23.1)	0.044
Grade 1–3	92 (89.3)	24 (96.0)	25 (92.6)	23 (92.0)	20 (76.9)	
Grade 0–1	41 (39.8)	9 (36.0)	6 (22.2)	11 (44.0)	15 (57.7)	0.047
Grade 2–3	62 (60.2)	16 (64.0)	21 (77.8)	14 (56.0)	11 (42.3)	
Grade 0–2	90 (87.4)	22 (88.0)	21 (77.8)	23 (92.0)	24 (92.3)	0.360
Grade 3	13 (12.6)	3 (12.0)	6 (22.2)	2 (8.0)	2 (7.7)	

Total skin toxicity	n (%)	HGF (pg/mL)				P -value
		≤ 1153.0	≤ 1337.1	≤ 1653.5	> 1653.5	
Grade 0	11 (10.7)	1 (3.8)	0 (0.0)	3 (11.5)	7 (28.0)	0.002
Grade 1–3	92 (89.3)	25 (96.2)	26 (100)	23 (88.5)	18 (72.0)	
Grade 0–1	41 (39.8)	10 (38.5)	5 (19.2)	9 (34.6)	17 (68.0)	0.019
Grade 2–3	62 (60.2)	16 (61.5)	21 (80.8)	17 (65.4)	8 (32.0)	
Grade 0–2	90 (87.4)	21 (80.8)	21 (80.8)	23 (88.5)	25 (100)	0.032
Grade 3	13 (12.6)	5 (19.2)	5 (19.2)	3 (11.5)	0 (0.0)	

Grades of total skin toxicity were divided into three patterns: grade 0/1–3; grades 0–1/2–3; and grades 0–2/3. When skin toxicity was divided into these grades, serum levels of amphiregulin (AREG), epiregulin (EREG), and hepatocyte growth factor (HGF) were inversely proportional to grades of total skin toxicity by the Cochran–Armitage test ($P < 0.05$). Serum level of HGF was inversely proportional to grades of total skin toxicity when skin toxicity was divided by all three patterns ($P < 0.05$). Significant P -values are indicated in bold.

AREG, EREG, and HGF were inversely proportional to grades of total skin toxicity ($P = 0.021$, $P = 0.047$, and $P = 0.019$, respectively). Serum HGF was also inversely proportional to grades of skin toxicity when grades of skin toxicity were divided into other patterns (grade 0/1–3, $P = 0.002$; grades 0–2/3, $P = 0.032$). Serum levels of EREG were inversely proportional to grades of skin toxicity when grades of skin toxicity were divided into grade 0 and grade 1–3 ($P = 0.044$). Serum levels of other ligands except for AREG, EREG, and HGF were evaluated, but there were no significant tendencies towards grades of skin toxicity by the Cochran–Armitage test. This result is summarized in Table S3.

In addition, we evaluated the association between serum levels of ligands and grades of acneiform eruption and paronychia by the Cochran–Armitage test. Serum levels of AREG tended to be inversely proportional to grades of acneiform eruption (grades 1–2/2–3, $P = 0.039$; grades 0–2/3, $P = 0.026$). In contrast, serum levels of EREG had tendencies to be inversely proportional to grades of paronychia (grade 0/1–3, $P = 0.044$). Serum levels of HGF were inversely proportional to grades of

both acneiform eruption (grade 0/1–3, $P = 0.012$) and paronychia (grade 0/1–3, $P = 0.005$; grades 0–1/2–3, $P = 0.039$).

Discussion

As in previous reports, our study revealed that clinical outcomes such as ORR, DCR, PFS, and OS were significantly better in patients with high grades of skin toxicity compared to those with low grades. As a novel finding, our study revealed that pretreatment serum levels of particular ligands such as HGF, EREG, and AREG were inversely proportional to the grades of total skin toxicity due to treatment with anti-EGFR antibodies when we divided the grade of skin toxicity into high grade and low grade.

Previous reports indicated that strong skin reactions due to anti-EGFR antibodies were associated with a better clinical outcome in patients with malignancies.⁽¹⁶⁾ This finding leads us to consider that dose escalation of anti-EGFR antibodies might improve the clinical outcome in patients with low grades of skin toxicities. The EVEREST study indicated that ORR

and DCR were significantly improved in patients who received escalating doses of cetuximab compared with those who received the standard dose⁽¹⁷⁾. In addition to CRC, the dose-escalation strategy for cetuximab in non-small-cell lung cancer patients was recently reported.⁽¹⁸⁾ The results of these clinical trials indicated that the dose-escalation strategy of anti-EGFR antibodies in patients with no or mild skin toxicity is one of the new strategies in malignant tumors, and further validation through large clinical trials are now ongoing in mCRC patients (EVEREST2, NCT01251536).

Among ligands that stimulate the ErbB family receptors, EREG is known as an autocrine growth factor in normal human keratinocytes, and organizes the epidermal structure by regulating keratinocyte proliferation and differentiation.⁽¹⁹⁾ In addition, EREG not only stimulates homodimers of both ErbB1 and ErbB4, but also activates all possible heterodimers of ErbB complexes. A previous report revealed that EREG played an autocrine role in the proliferation of human epithelial cells, presumably through cross-induction with other EGF family members.⁽²⁰⁾ Amphiregulin is a major autocrine factor for human keratinocytes. Expression of AREG is developmentally regulated in the epithelium and mesenchyme of human skin during morphogenesis.⁽²¹⁾ These broad biological activities of EREG and AREG may cause the difference in skin reactions by serum levels of these ligands under the suppression of EGFR.

Hepatocyte growth factor is an important factor in inducing motility in the corneal epithelium so wounds can be covered rapidly.^(22,23) In addition, Spix *et al.* previously reported that HGF induces epithelial cell motility through transactivation of the EGFR by the triple membrane-passing signaling mechanism in corneal epithelial cells. They also revealed that EGFR was also activated by HGF stimulation in human epidermal keratinocytes.⁽²¹⁾ These results showed that transactivation of EGFR by HGF-induced stimulation is a general phenomenon in epithelial cells.

Our study revealed that serum levels of HGF, EREG, and AREG were inversely proportional to the grade of skin toxicity, whereas other ligands had no significant association with the grade of skin toxicity. Epidermal keratinocytes are rich sources of EGFR ligands, including TGF- α , AREG, heparin binding-EGF, and EREG.⁽²³⁾ These ligands stimulate EGFR function to maintain cutaneous homeostasis, such as wound healing and EGFR-driven inflammatory reaction in keratinocytes.⁽⁸⁾ Particular ligands are strongly expressed in epithelium and cause various skin reactions in response to anti-EGFR antibodies by complex biological reactions and cross-talk of EGFR and c-MET signaling pathways under the suppression of EGFR.

Differences in grades of skin toxicity between acneiform eruption as acute toxicity and paronychia as late toxicity were observed in our present study. In addition, the serum AREG level was more closely associated with acneiform eruption than paronychia when compared with that of EREG and HGF. This may be associated with the change in serum levels of AREG during anti-EGFR antibody treatment. We previously evaluated not only pretreatment levels of ligands but also the change of serum ligands during anti-EGFR antibody treatment.⁽¹⁵⁾ Elevation of serum AREG levels during treatment was observed in 94.9% of patients, which may reduce the frequency and severity of paronychia. However, frequencies in the elevation of serum levels of HGF and EREG during treatment were lower compared with that of AREG.

Recently, Van Cutsem *et al.*⁽²⁴⁾ reported the clinical efficacy of combined treatment of fully monoclonal anti-HGF antibodies (rilotumumab and panitumumab) in *KRAS* WT CRC patients by a randomized phase Ib/II trial. In this study, panitumumab plus rilotumumab met the prespecified criterion for improvement in ORR, whereas any grades of rash, acneiform dermatitis, and paronychia were observed more frequently in patients who received a combination of rilotumumab and panitumumab compared with those who received panitumumab alone. This result indicates that low levels of both HGF and EGFR inhibition in cutaneous tissues may cause a more severe skin disorder, which is similar to the results of the present study. Severe skin toxicity causes some problems, such as reducing the patient's QOL and poor compliance with anti-EGFR antibody treatment. Further development of prevention and management for severe skin toxicity may solve these problems and improve the survival in mCRC patients undergoing anti-EGFR antibody treatment. Currently, several topical skin creams containing human growth factors or cytokines have been developed, mainly for cosmetics but also for skin disorders. These creams may be a novel approach to manage skin toxicity caused by a combination of anti-EGFR antibodies and ligand-targeted therapy.

Anti-EGFR antibodies are competitive ligand inhibitors against EGFR and the status of ligands that stimulate EGFR, c-MET, and IGF-IR are considered molecular markers to predict the efficacy or acquired resistance of anti-EGFR antibody treatment in solid tumors. In addition to gene mutations in the EGFR downstream signaling pathway, we previously reported that serum levels of particular ligands such as EREG and HGF were associated with prognosis and resistance to anti-EGFR antibody treatment in *KRAS* WT patients.⁽¹⁵⁾ Among several ligands, serum levels of HGF and EREG might play a significant role in predicting not only skin toxicity in cutaneous tissues, but also anticancer activity and chemotherapeutic resistance in tumor tissues during anti-EGFR antibody treatment.

There are several limitations in this study. First, we used serum samples that were obtained from residual blood samples and measurable items of ligands were limited. The validation of our findings in this present study is required by other clinical studies. Second, we evaluated the serum levels of ligands in the present study. Further investigation into the expression of ligands in cutaneous tissues could clarify the association between ligands and skin toxicity due to anti-EGFR antibodies.

In conclusion, the present study revealed that serum levels of HGF, EREG, and AREG at pretreatment were associated with skin toxicity grade in *KRAS* WT patients with mCRC. Recently, clinical trials investigating dual therapy using anti-EGFR antibodies and ligand-targeted antibodies are ongoing and under evaluation, whereas improvement in the management of skin toxicities are required to continue the treatment while maintaining patient QOL. Serum levels of these ligands may be significant markers to predict the grade of skin toxicity and the prognosis of anti-EGFR antibody treatment, which contribute to the improvement of skin toxicity and survival time.

Acknowledgments

We appreciate very much the participation of patients and their families in this study, and we thank all co-investigators for their contribu-

tions: Mrs. Mari Mizoguchi, Ms. Hideko Morita (ELISA analyses), and Mrs. Noriko Abe (serum preparation).

Disclosure Statement

Y.Y. has received honoraria from Taiho and Chugai, and research funding from Taiho, Chugai, Merck Serono, Astra Zeneca, and Daiichi-Sankyo. The other authors have no conflict of interest.

Abbreviations

AREG	amphiregulin
CI	confidence interval
CRC	colorectal cancer

DCR	disease control rate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EREG	epiregulin
HGF	hepatocyte growth factor
HR	hazard ratio
IGF-1	insulin growth factor-1
mCRC	metastatic colorectal cancer
NRG	neuregulin
ORR	objective response rate
OS	overall survival
PFS	progression-free survival
QOL	quality of life
TGF- α	transforming growth factor- α

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Progression-free survival (PFS) curves by grades of total skin toxicity.

Fig. S2. Survival curves of progression-free survival (PFS) and overall survival (OS) by grades of acneiform eruption (A, B) and paronychia (C, D) in KRAS WT patients.

Table S1. Protocols of ELISA kits for measurement of serum levels of each ligand

Table S2. Differences in grades of skin toxicity between acneiform eruption and paronychia

Table S3. Results of Cochran–Armitage test to evaluate the tendency between grades of skin toxicity and serum ligands (epidermal growth factor [EGF], transforming growth factor- α [TGF- α], neuregulin [NRG], and insulin growth factor-1 [IGF-1]).