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Serum levels of hepatocyte growth factor and epiregulin are associated with the prognosis on anti-EGFR antibody treatment in *KRAS* wild-type metastatic colorectal cancer

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Background: Ligands of transmembrane receptor tyrosine kinases have important roles in cell proliferation, survival, migration and differentiation in solid tumours. We conducted this study to evaluate the relationship between concentration of serum ligands and prognosis of patients with metastatic colorectal cancer (mCRC) treated with anti-epidermal growth factor receptor (EGFR) antibodies.

Methods: Between August 2008 and August 2011, serum samples were obtained from *KRAS* wild-type patients who met the inclusion criteria and received an anti-EGFR antibody treatment. Serum concentration of ligands was measured by an enzyme-linked immunosorbent assay, and somatic mutations of *KRAS*, *BRAF*, *PIK3CA* and *BRAF* were analysed by direct sequencing.

Results: A total of 103 patients were enrolled in the present study. At the pretreatment serum levels, patients with high levels of hepatocyte growth factor (HGF) had shorter progression-free survival (PFS) and overall survival (OS) compared with those with low levels of HGF (median PFS: 6.4 months *vs* 4.4 months; $P<0.001$, median OS: 15.3 months *vs* 8.0 months; $P<0.001$, respectively). Patients with high levels of epiregulin (EREG) also had shorter PFS and OS compared with those with low levels of EREG (median PFS: 6.6 months *vs* 4.9 months; $P=0.016$, median OS: 13.8 months *vs* 7.4 months; $P=0.048$, respectively). In addition, patients whose serum levels of ligands were elevated at progressive disease had shorter PFS and OS compared with other patients.

Conclusions: Our study indicated that high levels of HGF and EREG were associated with resistance to treatment with anti-EGFR antibodies in *KRAS* wild-type patients with mCRC. Our findings will contribute to the newly combination therapy on the treatment of anti-EGFR antibodies.

Colorectal cancer (CRC) is the second most common cause of cancer in women, the third most common in men and the fourth leading cause of cancer deaths worldwide (Jemal *et al*, 2011). Anti-EGFR monoclonal antibodies are active drugs, and gene mutations of *KRAS* codons 12 and 13 are recognised as strong predictive factors for no clinical benefit of anti-EGFR antibody treatment in mCRC (Jonker *et al*, 2007; Van Cutsem *et al*, 2009; Douillard *et al*, 2010). Several biomarkers for response to treatment with anti-EGFR antibodies have been investigated in mCRC. In particular, genomic changes in the EGFR downstream signal pathway, such as

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KRAS, *BRAF*, *PIK3CA* and *NRAS*, have been demonstrated to be associated with poor response and prognosis in mCRC patients who received anti-EGFR antibody treatment (Loupakis *et al*, 2010; De Roock W *et al*, 2010; Mao *et al*, 2012). However, these gene mutations are present at low frequency in mCRC, so further search for novel biomarkers is required.

EGFR is a receptor tyrosine kinase, activated by the binding of ligand to its extracellular domain, which triggers intracellular signalling through the *RAS/RAF/MAP*K and *PI3K/AKT* pathways that subsequently may modulate cell proliferation, adhesion, angiogenesis, migration and survival (Mendelsohn and Baselga, 2006; Scaltriti and Baselga, 2006). EGFR is a member of a family of related growth factor receptor tyrosine kinases that in addition to EGFR (ErbB1) include HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Eleven ligands have been identified in the ErbB family in humans: EGF, transforming growth factor-α (TGF-α), HB-EGF, betacellulin, amphiregulin (AREG), epiregulin (EREG), epigen and the neuregulins (NRG 1–4) (Yarden, 2001). Ligands binding to ErbB receptors induce the formation of receptor homodimers and heterodimers and activation of the intrinsic kinase domain, resulting in phosphorylation of specific tyrosine residues, which serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signal pathways (Hynes and Lane, 2005). EREG is known to bind more weakly to EGFR and ErbB4 than EGF but is much more potent than EGF and leads to a prolonged state of receptor activation (Shelly *et al*, 1998), but the molecular roles of EREG in cancer cells are not well known. Recently, some studies indicated that EREG and AREG expression of mRNA or immunohistochemistry (IHC) in tumour tissues was associated with response to and prognosis for the treatment with cetuximab in mCRC patients (Khambata-Ford *et al*, 2007; Jacobs *et al*, 2009). On the other hand, the clinical significance of serum levels of these ligands is still unclear with regard to the treatment with anti-EGFR antibodies in mCRC.

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchymal cytokine with a number of biological activities, including mitogenic, motogenic and/or morphogenic properties in epithelial tissues. HGF is also known as an angiogenesis factor by its ability to promote endothelial cell growth, survival and migration (Bussolino *et al*, 1992). HGF is a significant ligand of MET, which elicits multiple cellular responses regulating cell survival, morphogenesis, adhesion, migration, breakdown of extracellular matrix and angiogenesis (Birchmeier *et al*, 2003; Lesko and Majka, 2008). Recently, molecular target therapy directed at the HGF/MET signal pathway has been developed in solid tumours, and biomarkers for effectiveness of HGF/MET monoclonal antibody and tyrosine kinase inhibitor therapy are evaluated (Cecchi *et al*, 2012).

Use of anti-VEGF antibody (bevacizumab) as front-line therapy is the standard treatment for mCRC patients (Hurwitz *et al*, 2004; Saltz *et al*, 2008). A treatment that targets ligands of transmembrane receptor tyrosine kinases may be a promising approach in mCRC. We conducted this study to evaluate the relationship between serum concentrations of ligands and efficacy of anti-EGFR antibody treatment.

MATERIALS AND METHODS

Patients and sample collection. Between August 2008 and August 2011, specimens collected by endoscopic biopsy or surgical resection from 337 patients with advanced CRC were screened for the genomic status of *KRAS* codons 12 and 13 at the Gastrointestinal Oncology Division in National Cancer Center Hospital. We selected the mCRC patients who received anti-EGFR antibody treatment and whose tumours were *KRAS* wild-type (codons 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 at the division of clinical laboratories in National Cancer Center Hospital until use. We selected samples from these patients at two points as follows: (1) within 2 weeks before initiation of the treatment with anti-EGFR antibodies, and (2) within 2 weeks after diagnosis of progressive disease (PD) of anti-EGFR antibodies. In addition, we collected formalin-fixed paraffin-embedded (FFPE) tumour samples and performed genomic analyses of *KRAS* (codons 61, 146), *BRAF* (V600E), *PIK3CA* (exons 9, 20) and *NRAS* (codons 12/13, 61).

We enrolled the *KRAS* wild-type patients who met the inclusion criteria as follows: pathologically proven adenocarcinoma, metastatic or recurrent CRC, *KRAS* wild-type patients who had previously received one or more regimens of systemic chemotherapy, an Eastern Cooperative Oncology Group (ECOG) Performance status (PS) of 0–2, no significant abnormality of liver and renal function, patients who received combined chemotherapy or monotherapy with anti-EGFR antibodies, and who showed disease deterioration by computed tomography (CT) after anti-EGFR antibody treatment. Main exclusion criteria included the following: previous chemotherapy targeting the EGF pathway, other duplicated advanced cancer, and metastasis to central nervous system. Patients continued to receive chemotherapy until PD or intolerable toxicity from chemotherapy intervened. The response was evaluated by contrast-enhanced CT every 2–3 months. Patients' consent for the use of clinical materials was obtained, and this study was undertaken after approval by the institutional review boards.

ELISA. We chose ligands such as EGF, TGF-α, AREG, EREG, NRG, HGF and IGF-1, which was previously associated with resistance to target therapy for EGFR or HER2 in solid tumours.

HGF concentrations in serum were measured using a commercially available enzyme test (Human HGF Quantikine ELISA Kit, DHG00, R&D Systems, Minneapolis, MN, USA), and procedures were as follows: (1) we prepared all reagents, standard dilutions and samples as directed in the product insert, (2) added 150 μl of assay diluent to each well and then 50 μl of standard and sample to each well, (3) covered with a plate sealer and incubated at room temperature for 2 h, (4) aspirated each well and washed, repeating the process three times for a total of four washes, (5) we added 200 μl of HRP conjugate to each well, (6) covered with a new plate sealer, incubated at room temperature for 2 h and aspirated and washed four times, (7) we added 200 μl TMB substrate solution to each well and incubated at room temperature for 30 min with protection from light, (8) we added 50 μl of stop solution to each well, and (9) read at 450 nm within 30 min and set wavelength correction to 540 nm or 570 nm.

Concentrations of EREG in serum were measured using Human epiregulin ELISA kit (CSB-EL007779HU, CUSABIO, Wuhan, China). Procedures were as follows: (1) we prepared all reagents, standards and samples (5 × diluted with sample diluent), (2) added 100 μl of standard and sample per well and covered with a plate sealer and then incubated for 2 h at 37 °C, (3) removed the liquid from each well, (4) added 100 μl of Biotin-antibody (1 ×) to each well and covered with a plate sealer and then incubated for 1 h at 37 °C, (5) aspirated each well and washed, repeating the process two times for a total of three washes, (6) added 100 μl of HRP-avidin (1 ×) to each well and covered with a plate sealer and incubated for 1 h at 37 °C, (7) repeated the aspiration/wash process (for) five times, (8) added 90 μl of TMB substrate to each well and incubated for 15–30 min at 37 °C with protection from light, and (9) added 50 μl of Stop Solution to each well, read at 450 nm within 30 min and set wavelength correction to 540 nm or 570 nm.

We used ELISA kits to measure serum levels of ligands as follows: Uscn Life Science Inc. (Wuhan, China) (E90006Hu) for

AREG, R&D (DEG00) for EGF, R&D (DTGA00) for TGF-α, CUSABIO (CSB-E17153h) for NRG and R&D (DG00) for IGF-1.

Direct sequencing of *KRAS*, *BRAF*, *PIK3CA* and *NRAS*. DNA samples were extracted from FFPE tumour tissue sections. Tumour cell-rich area in the H/E section was marked under a microscope, and tissue was scraped from the corresponding area of another deparaffinised unstained section. DNA from the scraped-off tissue sample was isolated using the QIAamp DNA FFPE Tissue Kit (QIAGEN KK, Tokyo, Japan). Exon 2 (codons 12, 13), exon 3 (codon 61), exon 4 (codon 146) of *KRAS* gene and exon 15 (codon 600) of *BRAF* gene and exon 9 (codons 542, 545), exon 20 (codon 1047) of *PIK3CA* gene and exon 2 (codons 12, 13) and exon 3 (codon 61) of *NRAS* gene were amplified by PCR (the GeneAmp PCR System 9700 thermal cycler, Applied Biosystems, Foster City, CA, USA). The PCR products were visualised 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. To assess the associations of ligand protein with the objective response rate (ORR), disease control rate (DCR), progression-free survival (PFS) and overall survival (OS), the values for each ligand were categorised into low and high values with respect to the median. The efficacy consisted of RR, DCR, PFS and OS. Assessment of therapeutic response consisted of complete response (CR), partial response (PR), stable disease (SD), PD and not evaluated (NE), according to the Response Evaluation Criteria in Solid Tumors criteria ver. 1.0. ORR was defined as the proportion of patients whose best response was a CR or PR among all patients. DCR was defined as the proportion of patients whose best response was a CR, a PR or SD. PFS was defined as the interval from initiation of anti-EGFR therapy to the occurrence of PD or death without evidence of progression. OS was defined as the interval from initiation of anti-EGFR therapy to death or last follow-up.

Differences in the distribution of variables were evaluated using the Fisher's exact test or χ^2-test, as appropriate. PFS and OS were estimated by the Kaplan–Meier method, and survival curves were compared by the log-rank test. All tests were two-sided, and a P-value <0.05 was defined as statistically significant. We estimated hazard ratios (HRs) and the corresponding 95% confidence intervals (CIs) for PFS and OS using univariate and multivariate analysis by Cox proportional hazards model. Variables of patients' background included age, gender, ECOG PS, histological type, primary site, stage and metastatic sites. Receiver operating characteristics (ROC) curve analysis was performed to determine the optimal cutoff values for serum ligands as continuous variables.

We performed statistical analyses by the SPSS statistical software, version 19 (IBM, Tokyo, Japan).

RESULTS

A total of 113 *KRAS* wild-type patients met the selection criteria between August 2008 and August 2011 in our hospital. Background characteristics are summarised in Table 1. As previous chemotherapy, all patients received the standard fluoropyrimidine-based chemotherapy regimen, 112 of the 113 patients (99.0%) received oxaliplatin, and 94 of the 113 patients (91.3%) received irinotecan. Thirty-two patients (31.1%) received subsequent treatment after treatment with anti-EGFR antibodies. Nine patients were enrolled in phase I trials, and five patients received hepatic arterial infusion chemotherapy of 5-fluorouracil for liver metastases. Three patients received a combination of irinotecan plus mitomycin C, and one patient received immunotherapy. Fourteen patients received previous chemotherapy, which had been withdrawn due to intolerable adverse events.

Serum samples from 103 patients were used to measure the concentrations of ligands. Ten patients were excluded, because serum samples at two points were insufficient to analyse by ELISA. Results of serum levels of ligands are shown in Figure 1 (graphs) and Supplementary Data 1 (Table). We evaluated the prognostic role of these ligands by log-rank test. When the cutoff values set the median of serum levels of ligands, serum HGF and EREG were associated with prognosis on the treatment of anti-EGFR antibodies. Survival curves estimated by the Kaplan–Meier method are shown in Figure 2. We also evaluated the prognostic roles of other ligands, but there was no significant correlation between the serum concentration of each ligand (cutoff value: median) and clinical outcome such as PFS (Supplementary Data 2).

Table 1. Baseline characteristics of patients in this study

	Total
Number of patients	103
Median age (range), years	62.0 (26–81)
Gender (%)	
Male	65 (68.0)
Female	38 (32.0)
ECOG PS (%)	
0–1	97 (94.2)
2	6 (5.8)
Primary site (%)	
Colon	52 (50.5)
Rectum	51 (49.5)
Histological type (%)	
Well, mod	88 (85.4)
Por, sig	14 (13.6)
Muc	1 (1.0)
Number of metastatic site (%)	
1	29 (28.2)
⩽2	38 (71.6)
Treatment lines (%)	
Second	9 (8.7)
Third	67 (65.1)
⩽Fourth	27 (26.2)
Regimen of chemotherapy (%)	
Combination	82 (79.6)
Monotherapy	21 (20.4)
Anti-EGFR antibodies (%)	
Cetuximab	83 (80.6)
Patinimumab	20 (19.4)
Subsequent treatment (%)	
Yes	32 (31.1)
No	81 (68.9)

Abbreviations: ECOG PS = Eastern Cooperative Oncology Group Performance Status; EREG = epiregulin; HGF = hepatocyte growth factor; mod = moderately differentiated adenocarcinoma; muc = mucinous adenocarcinoma; por = poorly differentiated adenocarcinoma; sig = signet ring cell carcinoma; well = well differentiated adenocarcinoma. *P*-value: significant difference is <0.05.

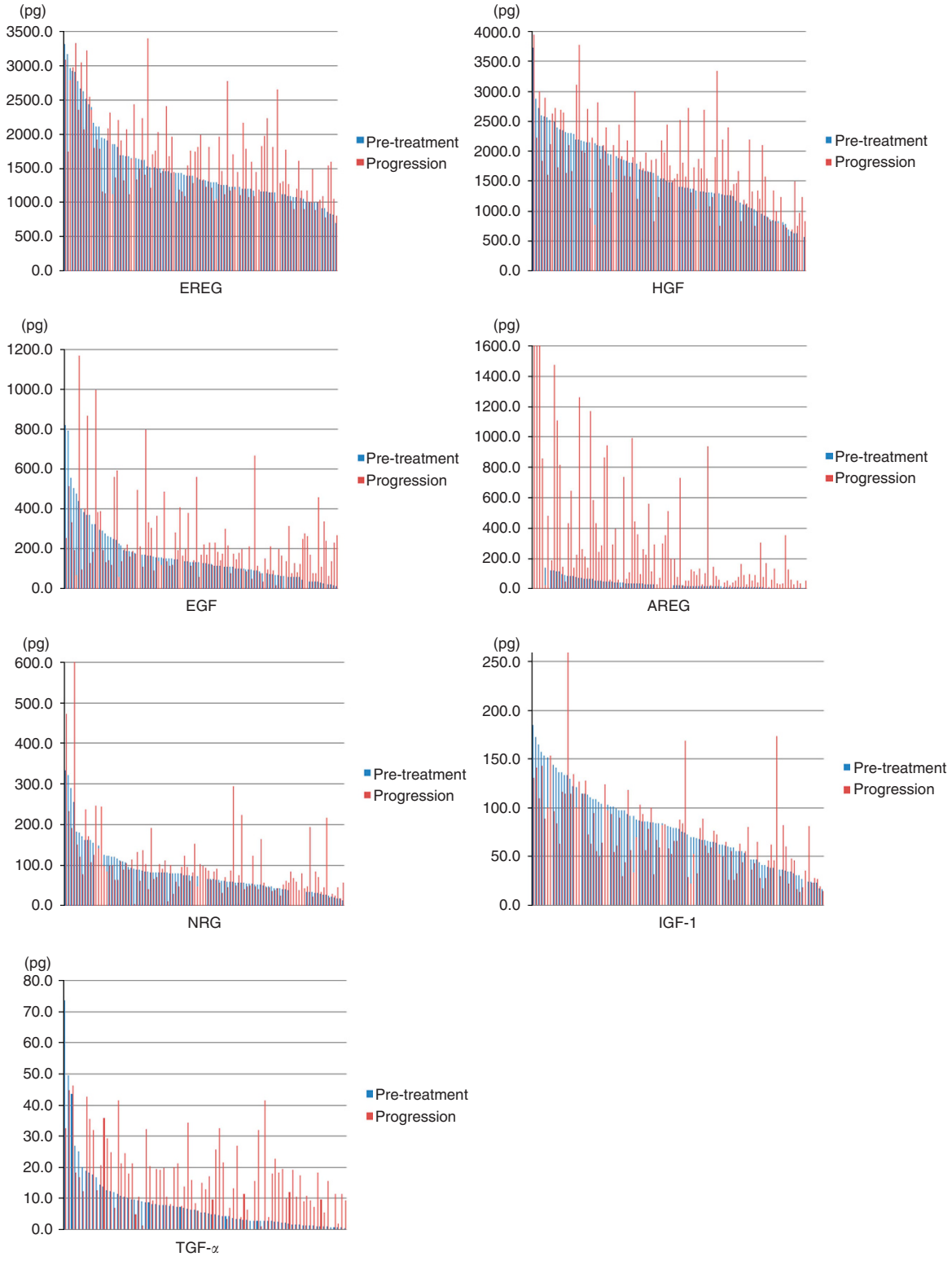

Figure 1. Graphical representations of serum concentrations of ligands measured by ELISA. Individual data of serum EREG, HGF, EGF, AREG, NRG, IGF-1 and TGF-α are summarized by graphs. Blue bars show serum levels at pre-treatment and red bars show those at progression disease.

We divided 103 patients into two subgroups of high and low levels, respectively, of the pretreatment serum concentrations of HGF and EREG. There were no significant differences in patients' characteristics between subgroups of each HGF and EREG levels (Supplementary Data 3).

Serum levels of HGF and EREG, genomic change of *KRAS*, *BRAF*, *PIK3CA* and *NRAS*. Serum concentrations of HGF and EREG were compared between subgroups of high/low levels and genomic mutations of *KRAS*, *BRAF*, *PIK3CA* and *NRAS* are shown in Table 2. The median serum concentration of HGF in patients whose pretreatment HGF levels were high was elevated at PD (pretreatment: 1672.5 pg ml^{-1}, PD: 1990.2 pg ml^{-1}). In contrast, in patients whose pretreatment HGF levels were low, the median HGF levels at PD were similar to pretreatment levels (pretreatment: 1154.8 pg ml^{-1}, PD: 1166.0 pg ml^{-1}). The median serum

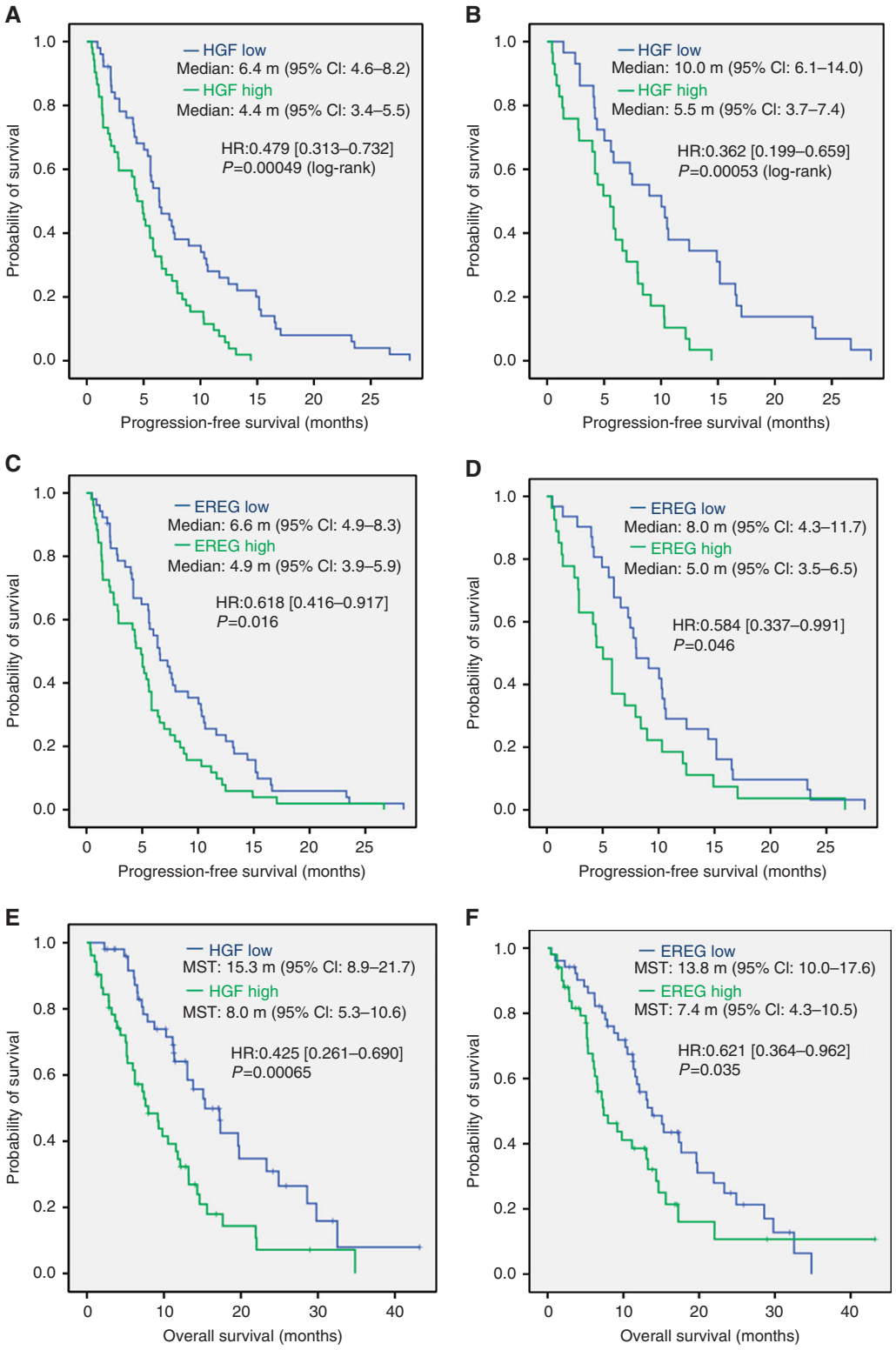

Figure 2. Survival curves of pretreatment hepatocyte growth factor (HGF) and epiregulin (EREG) levels among *KRAS* wild-type patients and all wild-type of *KRAS*, *BRAF*, *PIK3CA* and *NRAS*. Survival curves of PFS in terms of HGF levels are shown among (**A**) *KRAS* wild-type patients and (**B**) among all wild-type patients. Survival curves of PFS in terms of EREG levels are shown among (**C**) *KRAS* wild-type patients and (**D**) among all wild-type patients. Among *KRAS* wild-type patients, survival curves of OS in (**E**) HGF levels and (**F**) EREG levels are shown. Patients with low levels of ligands had longer OS compared with patients with high levels.

concentration of EREG was elevated at PD compared with pretreatment levels in patients whose pretreatment EREG levels were high (pretreatment: 1662.5 pg ml^{-1}, PD: 2188.4 pg ml^{-1}). The median of EREG levels at PD were similar to pretreatment levels in patients whose pretreatment EREG levels were low (pretreatment: 1,175.0 pg ml^{-1}, PD: 1,274.9 pg ml^{-1}). A total of 103 *KRAS* wild-type patients were investigated for genomic changes in *KRAS*, *BRAF*, *PIK3CA* and *NRAS*. Mutations of *KRAS* codon 61 ($N=2$, 1.9%), *KRAS* codon 146 ($N=5$, 4.9%), *BRAF* V600E ($N=2$, 1.9%), *PIK3CA* exon9 ($N=4$, 3.9%), *NRAS* codon

Table 2. Results of serum concentration of hepatocyte growth factor (HGF) and epiregulin (EREG) and genomic mutations of *KRAS*, *BRAF*, *PIK3CA* and *NRAS*

	Pretreatment HGF level		Pretreatment EREG level	
	High	**Low**	**High**	**Low**
Number of patients	52	51	51	52
Pretreatment serum concentration (pg ml^{-1})				
Median Range	1672.5 1361.8–3319.3	1154.8 703.7–1337.1	1662.5 1497.2–3731.5	1175 562.3–1485.2
Serum concentration at PD (%)				
Median Range	1990.2 1540.4–3391.1	1166 786.2–1532.4	2188.4 1725.8–3947.9	1274.9 582.9–1710.5
Change of serum levels after treatment				
Elevation (%) No elevation (%) Impossible to analyse (%)	25 (48.1) 27 (51.2) 0	35 (68.6) 16 (31.4) 0	28 (54.9) 22 (43.1) 1 (2.0)	31 (59.6) 21 (40.4) 0
Genomic mutations: total (%)	7 (13.4)	13 (25.5)	9 (17.6)	11 (21.1)
KRAS codon 61 (Q61H; $N=2$)	0	2 (4.0)	1 (2.0)	1 (1.9)
KRAS codon 146 (A146T; $N=4$, A146V; $N=1$)	1 (1.9)	4 (8.0)	1 (2.0)	4 (7.7)
BRAF codon 600 (V600E; $N=2$)	1 (1.9)	1 (2.0)	1 (2.0)	1 (1.9)
PIK3CA exon 9 (E545K; $N=3$, E545A; $N=1$)	1 (1.9)	3 (6.0)	2 (4.0)	2 (3.8)
PIK3CA exon 20	0	0	0	0
NRAS codon 12/13 (G13D: $N=2$)	1 (1.9)	1 (2.0)	1 (2.0)	1 (2.0)
NRAS codon 61 (Q61L; $N=3$, Q61E; $N=1$, Q61K; $N=1$)	3 (5.7)	2 (4.0)	3 (6.0)	2 (3.8)

Abbreviation: PD = progression disease.

12/13 ($N=2$, 1.9%), and *NRAS* codon 61 ($N=5$, 4.9%) were detected. In two patients, there were concomitant mutations of *KRAS* codon 146 and *PIK3CA* exon 9 and *NRAS* codon 61and *PIK3CA* exon 9. Patients with genomic mutations achieved no response to anti-EGFR antibodies (SD: $N=6$, PD: $N=12$).

PFS and OS by pretreatment levels of HGF and EREG in serum. Survival curves of PFS according to pretreatment levels of HGF and EREG are shown in Figures 1A–D. Among the 103 *KRAS* wild-type patients, patients with low levels of serum HGF had significantly longer PFS compared with those with high levels of serum HGF (median: 6.4 *vs* 4.4 months, HR: 0.479, 95% CI: 0.313–0.732, $P=0.00049$). Patients with low levels of serum EREG also had significantly longer PFS compared with those with high levels of serum EREG (median: 6.6 *vs* 4.9 months, HR: 0.618, 95% CI: 0.416–0.917, $P=0.016$). Among the 57 all-wild-type patients, patients with low levels of serum HGF had significantly longer PFS compared with those with high levels of serum HGF (median: 10.0 months *vs* 5.5 months, HR: 0.362, 95% CI: 0.199–0.659, $P=0.00053$). Patients with low levels of serum EREG also had significantly longer PFS compared with those with high levels of serum EREG (median: 8.0 *vs* 5.0 months, HR: 0.584, 95%CI: 0.337–0.991, $P=0.047$).

Survival curves of OS according to pretreatment serum levels of HGF and EREG are shown in Figures 1E and F. Among *KRAS* wild-type patients, those with low levels of serum HGF had significantly longer PFS compared with those with high levels of serum HGF (median: 15.3 *vs* 8.0 months, HR: 0.425, 95% CI: 0.261–0.690, $P=0.00065$). Patients with low levels of serum EREG also had significantly longer PFS compared with those with high levels of serum EREG (median: 13.8 *vs* 7.4 months, HR: 0.621, 95%CI: 0.364–0.962, $P=0.035$).

ORR and DCR by subgroups of HGF and EREG levels in serum. ORR and DCR of the treatment with anti-EGFR antibodies according to serum levels of HGF and EREG are shown in Table 3. Among *KRAS* wild-type patients who were enrolled in this study, 34 patients and 37 patients achieved PR and SD, respectively, and 32 patients had no response to anti-EGFR antibodies. ORR and DCR were 33.0% (95% CI: 24.1–43.0) and 68.9% (59.1–77.7), respectively. There was no significant difference in ORR between subgroups of high HGF levels and low HGF levels (30.8% *vs* 35.3%, $P=0.678$). There also was no significant difference, but a better response rate was observed in patients with low EREG compared with those in the high EREG group (40.4% *vs* 23.5%, $P=0.091$).

Among all-wild-type patients, ORR and DCR were 42.1% (95% CI: 29.1–55.9) and 80.7% (68.1–90.0%), respectively, which were better than the results for *KRAS* wild-type patients. There were significant better ORR and DCR in patients with low EREG compared with those with high EREG (ORR: 56.6 *vs* 25.9%, $P=0.035$; DCR: 93.3 *vs* 66.6%, $P=0.017$).

Prognosis by pretreatment levels of HGF and EREG and change of serum levels after treatment. Survival curves by pretreatment levels of HGF and EREG (high/low) and change of serum levels at PD compared with pretreatment (elevation/no elevation) levels are shown in Figure 3. In common for HGF and EREG, patients whose pretreatment levels were low and whose serum levels were not elevated at PD tended to have longer PFS and OS compared with other subgroups. Even if serum concentration at pretreatment was low, patients with elevated serum levels at PD tended to have shorter prognosis compared with patients whose serum levels were not elevated.

Table 3. Objective response rate (ORR) and disease control rate (DCR) by pretreatment serum levels of hepatocyte growth factor (HGF) and epiregulin (EREG) when the cutoff values are median of serum concentration

		Pretreatment HGF level (cutoff value: median)			Pretreatment EREG level (cutoff value: median)		
Response	**A total patients**	**High**	**Low**	***P*-value**	**High**	**Low**	***P*-value**
A							
PR	34 (33.0%)	16 (30.8%)	18 (35.3%)		11 (21.6%)	23 (44.2%)	
SD	37 (35.9%)	15 (28.8%)	22 (43.1%)		18 (35.3%)	19 (36.5%)	
PD	32 (31.1%)	21 (40.4%)	11 (21.6%)		22 (43.1%)	10 (19.2%)	
ORR (95% CI)	33.0% (24.1–43.0)	30.8% (18.7–45.1)	35.3% (22.4–49.9)	0.678	23.5% (12.8–37.5)	40.4% (27.0–54.9)	0.091
DCR (95%CI)	68.9% (59.1–77.7)	59.6% (45.1–73.1)	78.4% (64.7–88.7)	0.055	58.8% (44.2–72.4)	76.9% (63.2–87.5)	0.059
B							
PR	24 (42.1%)	9 (31.0%)	15 (53.6%)		7 (25.9%)	17 (56.6%)	
SD	22 (38.6%)	12 (41.4%)	10 (35.7%)		11 (40.7%)	11 (36.7%)	
PD	11 (19.3%)	8 (27.6%)	3 (10.7%)		9 (33.3%)	2 (6.7%)	
ORR (95% CI)	42.1% (29.1–55.9)	31.0% (15.3–50.8)	53.6% (33.9–72.5)	0.110	25.9% (11.1–46.3)	56.6% (37.4–74.5)	**0.031**
DCR (95%CI)	80.7% (68.1–90.9)	72.4% (52.8–87.3)	78.4% (71.8–97.7)	0.179	66.6% (46.0–83.5)	93.3% (77.9–99.2)	**0.017**

Abbreviations: CI = confidence interval; PD = progressive disease; PR = partial response; SD = stable disease. ORR and DCR in *KRAS* wild-type patients are shown in (A). ORR and DCR in all wild-type patients (*KRAS*, *BRAF*, *NRAS* and *PIK3CA*) are shown in (B). Bold values mean statistically significant difference (*P*-value <0.05).

Appropriate cutoff values of serum EREG and HGF that were evaluated by ROC curve analysis. Among *KRAS* wild-type patients, we evaluated the appropriate cutoff values of serum ligands to set it so that it became a DCR best. As a result, cutoff values of serum EREG and HGF were 1412.65 pg ml^{-1} (sensitivity: 0.719, specificity: 0.549) and 1393.55 pg ml^{-1} (sensitivity: 0.620, specificity: 0.656), respectively. At this cutoff values of serum EREG and HGF, ORR and DCR are summarised in Table 4. There were significant better ORR and DCR in patients with low EREG compared with those with high EREG (ORR: 43.8 *vs* 23.6%, $P=0.03$; DCR: 83.3 *vs* 56.4%, $P=0.003$). There was no significant difference in ORR, but significant better DCR in patients with low HGF compared with those with high HGF (DCR: 78.6 *vs* 57.4%, $P=0.021$). Survival curves of PFS are shown in Supplementary Data 4. Patients with low levels of serum ligands had significantly longer PFS compared with those with high levels of serum ligands.

Univariate and multivariate analyses in terms of PFS and OS in KRAS-WT patients. Results of univariate and multivariate analyses of prognosis in terms of PFS and OS are shown in Table 5. Multivariate analyses of our study showed that HGF and EREG levels in serum were prognostic factors in PFS (HR: 0.562, 95% CI: 0.591–0.878, $P<0.001$; HR: 0.646, 95% CI: 0.426–0.976, $P=0.039$, respectively). ECOG PS and HGF levels in serum were prognostic factors in OS (HR: 5.476, 95% CI: 2.029–14.776, $P<0.001$; HR: 0.447, 95% CI: 0.267–0.747, $P=0.002$, respectively).

DISCUSSION

Among the several ligands that stimulate EGFR, c-met and IGF-1 R, our study revealed that pretreatment serum concentrations of HGF and EREG had prognostic roles in the treatment with anti-EGFR antibodies in *KRAS* wild-type patients with mCRC. High levels of serum HGF and EREG at pretreatment were associated with shorter PFS and OS, whereas low levels of HGF and EREG were associated with better prognosis in the treatment with anti-EGFR antibodies. The response to anti-EGFR antibodies in terms of ORR and DCR showed no significant difference between serum levels of HGF and EREG but tended to be better in patients with low levels of serum HGF and EREG.

In the present study, we investigated the genomic mutations of *KRAS*, *BRAF*, *PIK3CA* and *NRAS*, because these mutations cause permanent activation of further downstream signal pathways and were known to be negative biomarkers for response to anti-EGFR antibody treatment (De Roock *et al*, 2010). In recent clinical trial of anti-EGFR antibodies in mCRC patients, *RAS* wild-type patients (*KRAS*, *NRAS*) are known to obtain clinical benefit of anti-EGFR antibodies compared with only *KRAS* wild-type patients. Among patients with all wild type of these mutations, we could separately evaluate the clinical significance of the predictive and prognostic roles of serum ligands. Actually, our results showed that high concentrations of serum HGF and EREG had significant prognostic value in predicting shorter PFS and OS in all wild-type patients. ORRs and DCR in patients with low levels of EREG were better than in patients with high levels of EREG.

To our knowledge, no previous studies in mCRC have evaluated the prognostic role of serum ligands in the treatment with anti-EGFR antibodies by monitoring changes in serum concentrations from initiation of treatment to diagnosis of PD. Our study revealed that elevation of serum concentrations of HGF and EREG at the time of PD were likely to associate with a poor prognosis. This may indicate that the serum levels of these ligands were associated with both early PD and delayed resistance to anti-EGFR antibodies. Previous reports show that the HGF/Met pathway was a significant factor in the development of resistance to EGFR-target therapy. For example, Wheeler *et al* (2008) reported that cetuximab-resistant cells manifested strong activation of ErbB2, ErbB3 and c-Met. EGFR upregulation promoted increased dimerisation with erbB-2 and erbB-3, leading to their transactivation. Yano *et al* (2008) reported that HGF induces EGFR-TKI resistance in EGFR mutant lung cancer. Engelman *et al* (2007) indicated that MET amplification causes gefitinib resistance by driving ErbB3-dependent activation of PI3K in non-small cell lung cancer (NSCLC). Xu *et al* (2010) indicated that EGFR regulates Met levels and invasiveness through hypoxia-inducible factor-1alpha in NSCLC. In CRC, Bardelli *et al* (2013) reported that the amplification of the MET receptor drives resistance to anti-EGFR therapies in CRC. These reports support that there is a cross-talk of signal pathways between members of the ErbB family and HGF/Met pathway.

Recently, phase I and II trials of HGF-targeted monoclonal antibodies, such as TAK701, AMG102 (rilotumumab) and

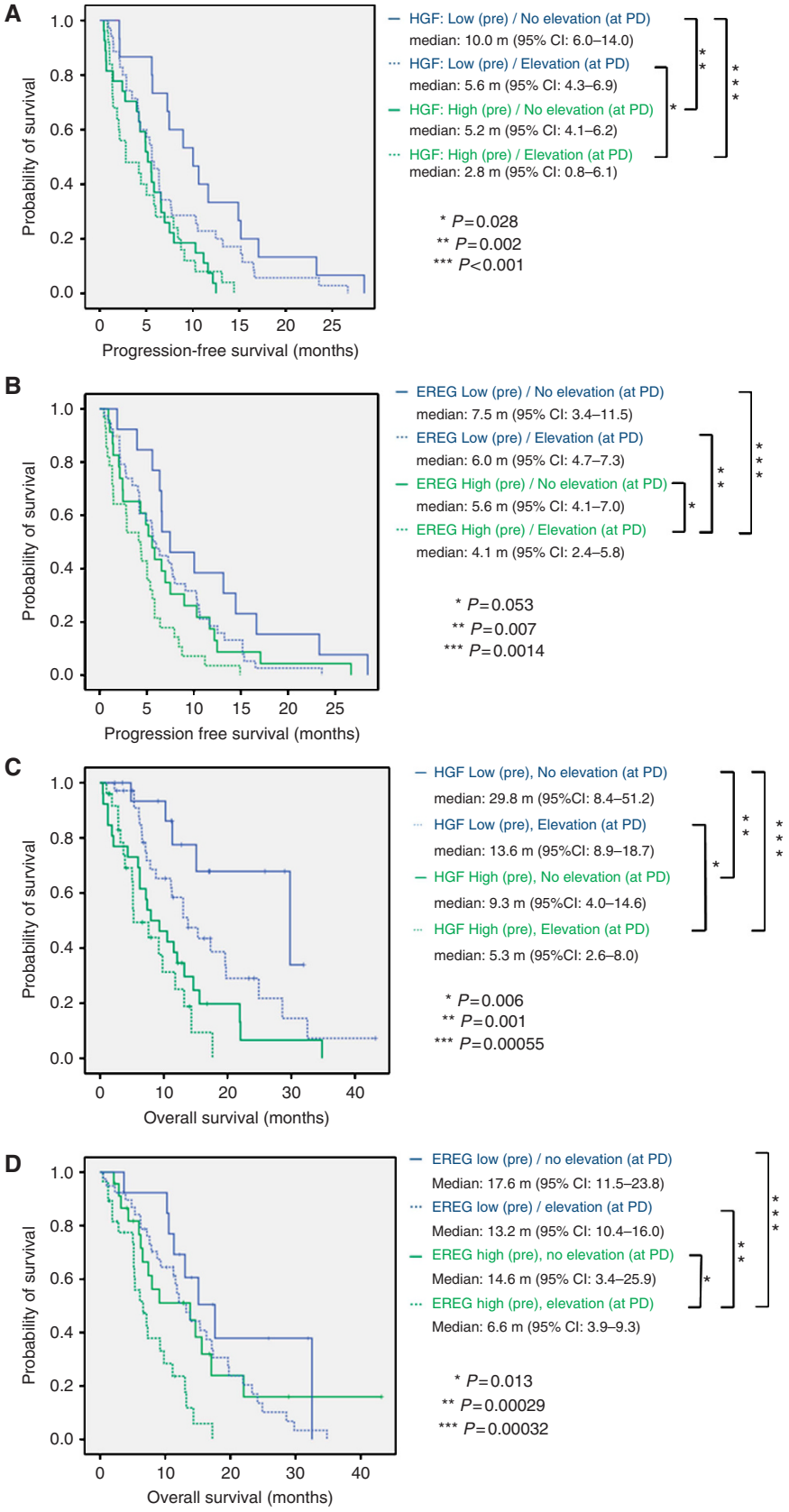

Figure 3. Survival curves by the change of serum levels of ligands after treatment. Survival curves of PFS by pretreatment levels of HGF and EREG (high/low) and change of serum levels at PD compared with pretreatment (elevation/no elevation) levels are shown. (**A**) PFS curves divided by change of serum HGF levels. (**B**) PFS curves divided by change of serum EREG levels. (**C**) OS curves divided by change of serum HGF levels. (**D**) OS curves divided by change of serum EREG levels.

SCH900105 (AV299), have been carried out in solid tumours (Meetze *et al*, 2009). A phase II randomised trial that compared rilotumumab or ganitumab (AMG 479) with panitumumab *vs* panitumumab alone in *KRAS* wild-type patients with mCRC revealed that the arm with rilotumumab achieved better response and PFS compared with panitumumab alone (Eng *et al*, 2011). In this trial, biomarker analyses of Met expression by IHC revealed that there was no association between efficacy and intensity of Met by IHC. Our data showing that high HGF levels in serum were associated with poor prognosis in the treatment with anti-EGFR

Table 4. Objective response rate (ORR) and disease control rate (DCR) by pretreatment serum levels of hepatocyte growth factor (HGF) and epiregulin (EREG) when the appropriate cutoff values were evaluated by ROC curve analysis

		Pretreatment HGF level (cut-off value: 1393.55 pg ml^{-1})			Pretreatment EREG level (cutoff value: 1412.65 pg ml^{-1})		
Response	**Total patients**	**High (*N* = 47)**	**Low (*N* = 56)**	***P*-value**	**High (*N* = 55)**	**Low (*N* = 48)**	***P*-value**
PR	34 (33.0%)	15 (31.9%)	19 (33.9%)		13 (23.6%)	21 (43.8%)	
SD	37 (35.9%)	12 (25.5%)	25 (44.7%)		18 (32.8%)	19 (39.5%)	
PD	32 (31.1%)	20 (42.6%)	12 (21.4%)		24 (43.6%)	8 (16.7%)	
ORR (95% CI)	33.0% (24.1–43.0)	31.9% (19.1–47.1)	33.9% (21.8–47.8)	0.829	23.6% (13.2–37.0)	43.8% (29.3–58.8)	**0.030**
DCR (95% CI)	68.9% (59.1–77.7)	57.4 % (42.2–71.7)	78.6% (65.6–88.4)	**0.021**	56.4% (42.3–69.7)	83.3% (69.8–92.5)	**0.003**

Abbreviations: CI = confidence interval; PD = progressive disease; PR = partial response; ROC = receiver operating characteristics; SD = table disease. Cutoff values were decided so that it became a DCR best. ORR and DCR in *KRAS* wild-type patients are shown. Bold values mean statistically significant difference (*P*-value <0.05).

Table 5. Univariate and multivariate analyses in *KRAS-WT* patients

(A)	PFS					
	Univariate analysis			Multivariate analysis		
Variables	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
ECOG PS						
0–1	1			1		
2	2.849	1.147–7.079	0.024	2.069	0.802–5.333	0.109
Gender						
Male	1					
Female	0.861	0.575–1.289	0.468			
Age						
⩽60	1					
>60	1.028	0.692–1.528	0.890			
Primary lesion						
Colon	1					
Rectum	1.080	0.729–1.598	0.702			
Histological type						
Well/mod	1					
Por/sig/muc	1.473	0.845–2.566	0.172			
Metastatic site						
1	1					
⩾2	1.041	0.676–1.603	0.856			
Serum HGF						
High	1			1		
Low	0.479	0.313–0.732	<0.001	0.562	0.359–0.878	0.011
Serum EREG						
High	1			1		
Low	0.618	0.416–0.917	0.017	0.646	0.426–0.979	0.039

Table 5. (Continued)

(B)

	OS					
	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	*P*-value	HR	95% CI	*P*-value
ECOG PS						
0–1	1			1		
2	6.637	2.611–16.870	<0.001	5.476	2.029–14.776	<0.001
Gender						
Male	1					
Female	1.377	0.833–2.275	0.212			
Age						
⩽60	1					
>60	1.200	0.744–1.936	0.455			
Primary lesion						
Colon	1					
Rectum	1.138	0.706–1.834	0.596			
Histological type						
Well/mod	1					
Por/sig/muc	1.804	0.929–3.503	0.081			
Metastatic site						
1	1					
⩾2	1.148	0.674–1.955	0.612			
Serum HGF						
High	1			1		
Low	0.425	0.261–0.690	<0.001	0.447	0.267–0.747	0.002
Serum EREG						
High	1			1		
Low	0.621	0.416–0.917	0.035	0.605	0.365–1.001	0.050

Abbreviations: CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group Performance Status; mod = moderately differentiated adenocarcinoma; muc = mucinous adenocarcinoma; PFS = progression-free survival; por = poorly differentiated adenocarcinoma; sig = signet ring cell carcinoma; well = well differentiated adenocarcinoma. Univariate and multivariate analyses in PFS and OS are shown in table (A) and (B), respectively.

antibody also support the ongoing clinical trials of HGF target therapy with a combination of anti-EGFR antibodies, and high HGF concentration in serum might be a promising biomarker for predicting prognosis.

EREG is mainly known as a ligand of EGFR and ErbB-4 and induces tyrosine phosphorylation of EGFR, ErbB-2, ErbB-3 and ErbB-4. Bio-pathological features and prognostic roles of EREG were evaluated in some solid tumours, such as oral squamous cell carcinoma, breast cancer, CRC and malignant fibrous histiocytoma (Yamamoto *et al*, 2004; Shigeishi *et al*, 2008; Révillion *et al*, 2008). In CRC, high levels of mRNA for EREG and AREG in tumour tissues at pretreatment are more likely to have anti-tumour activity resulting from cetuximab therapy (Khambata-Ford *et al*, 2007). This indicates EREG has a high dependency to EGFR and significant ligand for anti-EGFR antibodies treatment. In this report, there was no significant correlation between EREG protein in blood and mRNA levels in tumour tissues. The authors suggested that this might indicate the existence of posttranscriptional regulation of these genes. We thought that serum protein may also be affected by not only tumour tissues but also the normal tissues, and posttranslational modifications such as microRNAs or ubiquitination may be associated with serum levels of ligands (Lu *et al*, 2007; Mlcochova *et al*, 2013). From these considerations, mRNA in tumour tissue and levels of serous protein of these ligands may have the different meaning as biomarkers. Both tumour mRNA and serum protein are important to evaluate the mechanism of resistance of chemotherapy in any tumours. Next step, we need to validate the prognostic roles of both mRNA levels in tumour tissues and protein levels in serum on the anti-EGFR antibodies treatment.

Our study revealed that only EREG in ligands of ErbB family was significantly associated with prognosis in mCRC patients who received anti-EGFR antibodies. Anti-EGFR antibodies function as a competitive antagonist that inhibits the binding of ligands to EGFR. Roepstorff *et al* (2009) previously reported that EGFR ligands differentially affect EGFR endocytosis and recycling. The degree of competitive inhibition by anti-EGFR antibodies is considered to be different by ligand type. We would like to investigate the degree or change of competitive inhibition of EREG compared with other ligands by administrating the anti-EGFR antibodies in CRC cells. In addition, LGR5 – which is a Wnt target gene that has been reported to be a marker for normal stem cells in

the intestine – may be expressed in colon cancer cells and may be an important molecule to identify colon cancer stem cells (Vermeulen *et al*, 2010). Kobayashi *et al* (2012) indicated that CRC cells with expression of LGR5 interconvert to drug-resistant LGR5-negative cells after chemotherapy. EREG was expressed in these LGR5-positive and -negative cells, and the numbers of tumour cells were decreased by anti-EREG antibody treatment in metastatic models. These reports and our study support the notion that EREG has significant molecular roles and may be a promising molecular therapeutic target in mCRC patients.

The present study has limitations. At first, our study was retrospective, and patients whose blood samples were not stocked were excluded. Second, power is limited with 103 cases of available *KRAS* wild-type patients. The results in this study may be just due to lack of power. Further validation of the findings is needed through other prospective studies with inhibitors of these ligands.

In conclusion, the present study revealed that serum HGF and EREG levels were associated with resistance to anti-EGFR antibodies in *KRAS* wild-type patients with mCRC. Target therapy studies regarding the HGF/Met pathway are now ongoing and under evaluation. According to our data, the concentration of serum HGF might be a potential biomarker for prediction of response and prognosis in dual target therapy with anti-EGFR antibodies and HGF/Met inhibitors. The clinical significance of serum EREG in mCRC has been unclear so far, but our data suggest a novel therapeutic strategy of administration of dual monoclonal anti-EGFR antibodies plus EREG antibodies to mCRC patients with high EREG levels in serum or elevation of serum EREG levels after treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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