Amphiregulin/epiregulin mRNA Expression and Primary Tumor Location in Colorectal Cancer

HIDEKAZU KURAMACHI¹, GO NAKAJIMA², KAZUHIKO HAYASHI²,
TATSUO ARAIDA³ and MASAKAZU YAMAMOTO⁴

¹Department of Chemotherapy, Yachiyo Medical Center, Tokyo Women's Medical University, Chiba, Japan;

²Department of Chemotherapy and Palliative Care, Tokyo Women's Medical University, Tokyo, Japan;

³Department of Surgery, Yachiyo Medical Center, Tokyo Women's Medical University, Chiba, Japan;

⁴Department of Gastrointestinal Surgery, Tokyo Women's Medical University, Tokyo, Japan

Abstract. *Background/Aim:* Amphiregulin (AREG) and epiregulin (EREG) mRNA expression levels are predictors of response to anti-EGFR antibody therapy. Left-sided colon cancer is more sensitive to anti-EGFR antibodies than right-sided, although the mechanism is unclear. The aim of this study was to determine the relationship between AREG, EREG mRNA expression levels and tumor location as well as the efficacy of anti-EGFR antibody agents. *Materials and Methods:* Real-time PCR was used to assess AREG and EREG mRNA expression in metastatic colorectal cancer (CRC) samples from 153 patients. *Results:* Among KRAS^{wt} samples, high AREG expression (AREG^{High}) was significantly more common in left-sided tumors than in right-sided. Among patients who received anti-EGFR antibody, response rates were significantly higher in AREG^{High} than in AREG^{Low}. In the left-sided tumor group, overall survival was significantly longer in patients with high EREG levels than with low levels, whereas the right-sided tumor group showed no survival difference between them. *Conclusion:* AREG and EREG mRNA expression levels in left-sided CRC were higher than in right-sided tumors. This may help explain why left-sided CRC is more responsive to anti-EGFR antibodies.

Amphiregulin (AREG) and epiregulin (EREG) belong to the epidermal growth factor (EGF) family, and act as mitogenic stimulators through binding to EGFRs (1). Recently, patients with high AREG and EREG expression in tumor cells were

reported to respond better to anti-EGFR antibody agents (cetuximab [CTX]/panitumumab [Pmab]; C/P) and survive longer than those with low expression among patients with unresectable/metastatic RAS-wild-type colorectal cancer (CRC) (2-13). Thus, AREG and EREG are potential alternative biomarkers for anti-EGFR antibodies.

Tumor location is also associated with prognosis in CRC. Patients with left-sided tumors show a better survival than those with right-sided tumors, regardless of therapy (14). Tumor location is also associated with response to C/P. In the CALGB 80405 study, among patients with RAS/BRAF-wild-type tumors, in the subset with left-sided CRC, those treated with CTX had 2 months' gain in overall survival (OS) compared to those treated with bevacizumab, whereas in the right-sided subset, patients treated with CTX showed 16 months' loss of OS compared to those treated with bevacizumab (15). Although many studies support the consensus that anti-EGFR antibody agents are more effective on left-sided tumors than right-sided ones, (16-19) the reason for this difference of efficacy is unclear. Lee *et al.* reported that EREG and AREG expression was inversely correlated with both methylation and right-sidedness in primary tumors, which suggests that these differences are related to the association between tumor location and C/P efficacy (20).

In this study, we compared mRNA expression levels of AREG and EREG in left-sided and right-sided tumors in 153 CRC patients, and evaluated the association between AREG and EREG expression and tumor location with efficacy of C/P in 49 patients.

Materials and Methods

Patients and samples. We analyzed data and specimens from 153 patients with metastatic or relapsed CRC who had undergone resection of primary colorectal adenocarcinomas between 2003 and 2013 in the Department of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan (n=136), or Tokyo Women's Medical University, Yachiyo Medical Center, Chiba, Japan (n=17).

Correspondence to: Hidekazu Kuramochi, Tokyo Women's Medical University, Yachiyo Medical Center, Department of Surgery, 477-96 Owadashinden, Yachiyoshi, Chiba 276-8524, Japan. Tel: +81 474506000, Fax: +81 474587047, e-mail: kuramochi.hidekazu@twmu.ac.jp

Key Words: Amphiregulin (AREG), epiregulin (EREG), colorectal cancer, tumor location, anti-EGFR antibody.

The patients' relevant characteristics are shown in Table I. Of the 153 samples, 100 were *KRAS* exon 2 (codon 12, 13) wild-type (*KRAS*^{wt}) and 53 were mutant type (*KRAS*^{Mut}). Of these 153 patients, 49 patients (*KRAS*^{wt}: n=37; *KRAS*^{Mut}: n=10; unknown: n=2) had received anti-EGFR antibodies (C/P).

Primary specimens were classified as having right-sided CRC if they were located in the cecum, ascending colon, hepatic flexure, or transverse colon, and left-sided CRC if the tumor site was within the splenic flexure, descending colon, sigmoid colon, or rectum.

This study was approved by the Ethics Committee of Tokyo Women's Medical University and performed in accordance with the Declaration of Helsinki. All patients were Japanese, and all had given their written informed consent according to the institutional regulations.

Microdissection. Formalin-fixed, paraffin-embedded tumor specimens were cut into 10-μm-thick serial sections. For pathological diagnosis, one slide for each specimen was stained with hematoxylin and eosin and evaluated by a pathologist. Manual microdissection using a scalpel was performed if histology was homogeneous and contained >90% cancer cells. For all other samples, laser-capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) was performed to ensure that only tumor cells were dissected.

RNA isolation and cDNA synthesis. Isolation of RNA from formalin-fixed paraffin-embedded (FFPE) specimens was performed using a RNeasy FFPE Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. We converted cDNA from the total RNA yielded, using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan).

Reverse transcription-PCR. cDNA was pre-amplified using a TaqMan PreAmp Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions. mRNA expression of *AREG*, *EREG*, and a single internal reference gene (*beta-2-microglobulin*) was measured by a fluorescence-based real-time polymerase chain reaction (PCR) detection method. Primers and probes were provided from TaqMan Gene Expression Assays (Applied Biosystems). The PCR Primer ID were *AREG*: Hs00950669_m1, *EREG*: Hs00914312_m1, and *beta 2-microglobulin*: Hs99999907_m1. The PCR methodology has been described previously (21). The 2^{-ΔΔCT} number was used for relative mRNA quantification. The samples from Tokyo Women's Medical University Hospital were measured using StepOne real-time PCR system (Applied Biosystems), and the samples from Yachiyo Medical Center were measured using Illumina ECO Real-time PCR system (Illumina, San Diego, CA, USA). Because PCR machines differed between the two institutes, median values of *AREG* and *EREG* expression in each institute were used as cut-off values, to divide high and low expression.

Screening for *KRAS* mutation. DNA was extracted from the FFPE specimens using a Qiaamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. *KRAS* exon-2 mutations were detected by direct-sequencing, as previously described (22).

Statistical analysis. Frequencies of high and low gene expression were compared between left- and right-sided tumors using Fisher's exact test. Response rates and carcinoembryonic antigen (CEA) changing rates were compared between high- and low-gene

Table I. Demographic and clinical parameters of 153 patients with metastatic colorectal cancer.

Characteristics	Frequency	%
Age		
Mean (range)	64 (32-91)	
Gender		
Male	89	58.2%
Female	64	41.8%
Anatomical site		
Cecum	13	8.5%
Ascending	31	20.3%
Transverse	9	5.9%
Descending	7	4.6%
Sigmoid	54	35.3%
Rectum	39	25.5%
<i>KRAS</i> exon 2 mutational status		
Wild-type	100	65.4%
Mutant	53	34.6%
Institution		
Tokyo Women's Medical Univ.	136	88.9%
Yachiyo Medical Center	17	11.1%
Received anti-EGFR therapy		
Received	49	32.0%
Not received	104	68.0%

Table II. Demographic and clinical parameters of 37 patients with *KRAS* wild-type metastatic colorectal cancer treated with anti-EGFR therapy.

Characteristics	Frequency	%
Age		
Mean (range)	67 (48-78)	
Gender		
Male	21	56.8%
Female	16	43.2%
Anatomical site		
Cecum	3	8.1%
Ascending	6	16.2%
Transverse	2	5.4%
Descending	2	5.4%
Sigmoid	12	32.4%
Rectum	12	32.4%
Treatment line		
1st line	3	8.1%
2nd line	13	35.1%
3rd line	21	56.8%
Regimen		
Cmab alone	2	5.4%
Cmab + CPT-11	14	37.8%
Cmab + FOLFOX	4	10.8%
Cmab + FOLFIRI	3	8.1%
Pmab alone	4	10.8%
Pmab + CPT-11	2	5.4%
Pmab + FOLFIRI	8	21.6%

Cmab: Cetuximab; CPT-11: irinotecan; Pmab: panitumumab.

Figure 1. Percentages of *AREG*^{High} patients by tumor side among *KRAS* wild-type and *KRAS*-mutant samples. Among *KRAS* wild-type patients, the percentage of *AREG*^{High} patients was significantly higher in the left-sided subset than in the right-sided subset.

expression groups using the Wilcoxon signed-rank test. The Kaplan–Meier method was used to construct survival curves and the log-rank test for statistical analysis. The Cox proportional hazard regression model was used in multivariate analysis.

OS was defined as the time from the first administration of anti-EGFR antibodies to death from any cause. CEA changing rate was defined as minimum serum CEA levels minus baseline CEA levels, divided by baseline CEA levels. Statistical analyses were performed using JMP 11 (SAS Institute, Cary, NC, USA). *p*-Value <0.05 was considered significant. All values are two-sided.

Results

***AREG/EREG* mRNA expression and tumor location.** In the cohort as a whole, *AREG/EREG* mRNA expression levels did not significantly differ between left and right CRC. However, among *KRAS*^{wt} samples (n=100), *AREG*^{High} expression was significantly more common in left-sided CRC (high: n=41, low: n=28) than in right-sided CRC (high: n=9, low: n=22; *p*=0.0089; Figure 1). Similar results were observed with *EREG* expression, although not significantly so (left–high: n=39, low: n=30; right–high: n=11, low: n=20; *p*=0.08). However, among *KRAS*^{Mut} patients (n=53), no association was

observed in *AREG* or *EREG* mRNA expression levels between left- and right-sided tumors (*AREG*: left–high: n=13, low: n=18; right–high: n=11, low: n=11; *p*=0.58. *EREG*: left–high: n=12, low: n=19; right–high: n=8, low: n=14; *p*=1.00).

***AREG/EREG* mRNA levels and response to anti-EGFR antibodies.** Of the 153 patients, 49 received anti-EGFR antibodies (CTX/Pmab). As *KRAS* testing was only approved in Japan in 2010, 10 patients who were treated before approval had *KRAS*^{Mut} tumors and two patients had unknown *KRAS* status; the other 37 patients had *KRAS*^{wt} tumors (Table II). Twenty-three patients received CTX-containing regimens, and 14 were administered Pmab regimens, mostly as 2nd- or 3rd-line treatments.

Response rates (RRs) for *KRAS*^{wt} tumors were: 32.4% (12/37), *KRAS*^{Mut} tumors: 0% (0/10). In the *KRAS*^{wt} group (n=37), RRs significantly differed between the *AREG*^{High} group (47.6%, 10/21), and the *AREG*^{Low} group (12.5%, 2/16; *p*=0.035), where the median value from each testing institution was used as the cut-off. RRs by *EREG* expression showed a similar trend (*EREG*^{High}: 40.9%, *EREG*^{Low} group: 20%), but was not significant (*p*=0.28).

Figure 2. Overall survival (OS) in patients by AREG expression and EREG expression in patients who received anti-EGFR therapy. EREG expression levels clearly separated the patients with good and poor prognosis.

CEA changing rate was defined as $([\text{minimum serum CEA}] - [\text{baseline CEA}]) / (\text{baseline CEA})$. Median CEA changing rate in the $AREG^{High}$ group (-0.63) was significantly lower than in the $AREG^{Low}$ group (-0.13 ; $p=0.027$); and significantly lower in the $EREG^{High}$ group (-0.22) than in the $EREG^{Low}$ group (0.035 ; $p=0.0038$).

AREG and EREG mRNA levels and OS in C/P-treated patients. For all 49 C/P-treated patients, median survival time (MST) was significantly longer in the $EREG^{High}$ group (735 days) than in the $EREG^{Low}$ group (193 days, $p=0.0008$; hazard ratio [HR]=0.30; 95% confidence interval [CI]=0.13–0.62; Figure 2). This difference was even more pronounced in the $KRAS^{wt}$ group ($n=37$; MST: not reached vs. 169 days, $p=0.0035$, HR=0.29; 95%CI=0.12–0.70).

AREG showed a similar trend for MST, but was not significant (all patients: $AREG^{High}$: 552 days, $AREG^{Low}$: 197 days, $p=0.09$, HR=0.60, 95%CI=0.24–1.62; Figure 2; $KRAS^{wt}$: $AREG^{High}$: 735, $AREG^{Low}$: 193 days, $p=0.17$, HR=0.56, 95%CI=0.23–1.32).

Figure 3 shows a Kaplan–Meier curve for OS that combines KRAS and EREG status. The $KRAS^{wt}/EREG^{High}$ group had the most favorable outcome (MST: not reached). Interestingly, MST in the subgroup of $EREG^{Low}/KRAS^{wt}$ patients who were treated with C/P (169 days) was fairly similar to that of the

$KRAS^{Mut}$ group (226 days; Figure 3), which implies that the $EREG^{Low}$ patients derived no benefit from C/P.

C/P response by tumor location and AREG/EREG mRNA levels. We considered tumor location in $KRAS^{wt}$ tumors treated with anti-EGFR antibodies ($n=37$). The proportion of $AREG^{High}$ tumors was significantly higher in left-sided CRC (73%) than right-sided CRC (18%, $p=0.003$). EREG showed a similar trend, but was not significant (left: 69%, right: 36%, $p=0.06$).

Among patients with left-sided CRC, RRs to C/P in the $AREG^{High}$ group ($n=19$) were 47.4%, and in the $AREG^{Low}$ group ($n=7$) 28.6%. Among those with right-sided tumors, the $AREG^{High}$ group ($n=2$) included one responding patient (50%), whereas no patients in the $AREG^{Low}$ group ($n=9$) showed any response at all (0%).

Tumor location and OS in patients treated with C/P. In the $KRAS^{wt}$ group, among patients treated with C/P ($n=37$), MST was longer in the left-sided group (735 days) than in the right-sided group (169 days), but was not significant ($p=0.13$). In the left-sided CRC ($n=26$), MST was significantly longer in the $EREG^{High}$ group (MST: not reached) than in the $EREG^{Low}$ group (199 days; $p=0.018$; Figure 4). The $EREG^{High}$ /left-sided group had the most

Figure 3. Overall survival (OS) by KRAS status and EREG expression in patients who received anti-EGFR therapy. The KRAS-wild-type/*EREG*^{High} group had the most favorable outcome, whereas OS in the KRAS wild-type/*EREG*^{Low} group was similar to the KRAS-mutant group.

favorable outcome, whereas OS in *EREG*^{Low} group, even with left-sided CRC, was similar to those with right-sided CRC. In multivariate analysis, only *EREG* expression was an independent prognostic indicator ($p=0.0088$), whereas *AREG* expression ($p=0.41$), tumor sidedness ($p=0.52$), age (cut-off: 65 years; $p=0.98$), sex ($p=0.18$), and use of C/P ($p=0.93$) were not significantly relevant.

Discussion

Many recent studies support the association of tumor sidedness with response to C/P by metastatic CRC (16-19). Arnold *et al.* reported that OS and RR of patients who received anti-EGFR antibodies were significantly higher than those who did not receive anti-EGFR antibodies among patients with left-sided primary tumors, whereas no benefit from C/P was observed in patients with right-sided tumors by meta-analysis of six clinical trials (16). However, the mechanism of this phenomenon is unclear.

In the present study, our data showed that high *AREG* mRNA expression is significantly more common in left-

sided tumors than right-sided tumors. Lee *et al.* reported that *AREG/REG* expression was highly inversely correlated with the methylation of loci within the *AREG/REG* promoters, and was inversely correlated with right-sided tumors and CpG island methylator phenotype (CIMP) status (20). They also reported that high CIMP status is associated with inferior progression-free survival (PFS) after C/P treatment (20), although they did not directly evaluate *AREG/REG* expression and PFS. Our data are compatible with their report, and directly show the association between tumor location, *AREG/REG* expression, and prognosis of patients who received C/P, which implies that these factors are connected to the underlying mechanism of why C/P is more effective in left-sided primary tumors than in right-sided tumors. Although our study cohort was relatively small, one patient in the *AREG*^{High}/right-sided tumor group responded to C/P, although there were no responders in the *AREG*^{Low}/right-sided tumor group. Even among patients with left-sided tumors, OS of the *EREG*^{Low} subgroup was similar to that in the right-sided tumor group. As variations in response to C/P reflect differences in molecular profiles

Figure 4. Overall survival (OS) by EREG expression and tumor sidedness among patients who received anti-EGFR therapy. The $EREG^{High}/left$ -sided group had the most favorable outcome, whereas among the right-sided group, $EREG^{Low}$ and $EREG^{High}$ patients had similar outcomes.

such as *AREG/EREG* expression, tumor location may be just a surrogate marker of these differences.

High *AREG/EREG* expression is associated with better outcome in C/P-treated patients with CRC. Khambata-Ford *et al.* used microarrays to compare the expression of various genes between a disease control group and a non-responders group in a cohort of 80 CRC patients who received CTX monotherapy; *AREG* and *EREG* showed significant differential expression, with the lowest p-values (2). They showed that CTX-treated patients with high *EREG*- or *AREG*-expressing tumors have significantly longer PFS than patients with low expression. Jacobs *et al.* found that *EREG* and *AREG* expression was significantly associated with RR, disease control, PFS, and OS in 121 patients with irinotecan-refractory, *KRAS*^{wt} metastatic CRC who received a combination of CTX and irinotecan (3). Several other reports showed similar results; high *AREG* and/or *EREG* expression were related to better response for patients who received C/P (4-13). Similarly, our study also showed the patients with high *AREG/EREG* had better RR and longer OS than those with low levels among CP-treated patients with *KRAS*^{wt} CRC. These results were reproducible in every reported study, which indicates that *AREG* and *EREG* could be reliable biomarkers when combined with *RAS/BRAF* mutation status.

In contrast to these investigations, two studies on first-line treatments failed to show the benefit of *AREG* and *EREG* as predictive biomarkers in CRC in patients treated with CTX combined with multiple cytotoxic agents (9, 23). Cytotoxic chemotherapy shows promising effect along with biological agents in first-line settings, demonstrating remarkable additional survival benefit with biological agents may be difficult. The CRYSTAL trial, which compared FOLFIRI + CTX vs. FOLFIRI alone in the first-line setting, showed only a 0.9-month median gain in PFS (24), whereas late-line use of biological agents showed longer survival gains with remarkable hazard ratio improvement (25, 26). Where our study also showed remarkable OS separation by *EREG* expression, most of our cohort patients in our study received anti-EGFR agents as second- or third-line treatments. Thus, these reports (9, 23) do not show *AREG* and *EREG* to be equivocal predictive markers for C/P response in the first-line setting. Sunakawa *et al.* showed the effectiveness of *AREG* as a predictive biomarker for response to CTX in a first-line setting (11).

Our study showed that *EREG* was more likely to affect survival time, whereas *AREG* was more likely to affect response rate. *AREG* and *EREG* are both located in 4q13.3 chromosome, and their expression levels were strongly

correlated. We previously reported strong correlations between *AREG* mRNA expression and *EREG* mRNA expression ($R_s=0.82$, $p<0.0001$, Spearman correlation test) (21). Thus, *AREG*^{High} tumors are also likely to be *EREG*^{High}. Oliveras-Ferraros *et al.* reported that stable silencing of either *AREG* or *EREG* was found to reduce the expression of the other ligand in a squamous cell carcinoma cell line (27). As their expression is strongly correlated, the significance of *AREG* and *EREG* as biomarkers might not intrinsically differ.

This study has several limitations. First, this was a retrospective observational study with somewhat diverse patient backgrounds, relatively few patients received C/P, tissue collection timing varied, and minor *RAS* and *BRAF* mutations were not examined. Although many previous studies report *AREG* and *EREG* to be promising biomarkers for predicting response to C/P, several problems must be overcome to make them practicable. Most previous studies measured *AREG/EREG* mRNA expression as biomarkers, either using microarray or real-time RT-PCR. When quantitative PCR is used, relative quantitative values may change under various conditions, such as probe/primer design, length of amplicon, internal reference genes, types of PCR machines and protocols. Standardizing these conditions among various laboratories may be difficult. Optimal cut-off values are also undetermined. For quality control, standardization of methods for RNA extraction, reverse transcription, and PCR conditions are critical for obtaining consistent and reliable gene expression measurement for clinical use.

In conclusion, *AREG* and *EREG* expression was a reliable predictor of response to C/P, and was higher in left-sided CRC than in right-sided CRC. Although these data may help explain why C/P is more effective in left-sided tumors than in right-sided tumors, further studies are warranted to elucidate the mechanism of this phenomenon.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this study.

Author's Contributions

H.K. and G.N. designed and performed experiments, analyzed the data. H.K. wrote the manuscript. K.H., T.A., and M.Y. organized the study, and reviewed and edited the manuscript.

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