Published in final edited form as:

Pharmacogenet Genomics. 2014 November ; 24(11): 539–547. doi:10.1097/FPC.0000000000000087.

Single nucleotide polymorphisms in AREG and EREG are prognostic biomarkers in locally advanced gastric cancer (GC) patients after surgery with curative intent

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Abstract

Objective—Amphiregulin (AREG) and epiregulin (EREG) are important ligands to the epithelial growth factor receptor (EGFR) which is involved in the regulation of progression and stemness in gastric cancer (GC). This study investigated whether frequent single nucleotide polymorphisms (SNPs) in genes of *AREG* and *EREG* are associated with recurrence-free survival and overall survival in patients with locally advanced gastric cancer (GC).

Methods—SNPs with a minor allele frequency of 10% were analyzed using direct DNA sequencing in two independent study populations.

Results—The minor allele of *AREG* rs1615111 was associated with a significant higher 3-year recurrence rate and lower 3-year survival rate (HR= 2.21 and 2.35 respectively) when compared to patients homozygous for the dominant allele G. The value for overall survival could be validated with a HR of 2.54 ($P=0.018$) in an independent cohort. Patients homozygous for the minor allele A of *EREG* rs12641042 had a significant higher 3-year survival rate than patients having allele C (HR 0.48; $P=0.034$), but significance was lost in multivariable analysis ($P=0.066$). Value of rs12641042 could not be validated ($P=0.98$). Exploratory multivariable subgroup analysis

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Conflict of interest:

The authors declare to have no conflict of interest.

revealed the strongest prognostic value for rs1615111 in tumors with a diffuse histology ($P_{\text{for interaction}} = 0.004$).

Conclusions—*AREG* rs1615111, located in the *AREG* genomic region is able to significantly define different prognostic cohorts in locally advanced GC. This value is most evident in GC patients with diffuse histology which might be relevant as none of the trials testing EGFR-inhibitors has been enriched for diffuse histology or a molecularly defined population.

Keywords

gastric cancer; prognosis; single nucleotide polymorphism; amphiregulin

Introduction

Gastric cancer (GC) is one of the most common and deadliest solid malignancies worldwide with an estimated 22,220 new cases and an estimated 10,990 cancer related deaths in the US in 2014 [1]. The variety of the molecular makeup of GC is reflected by the regional differences in frequency, type and prognosis of GC. In the United States the age-adjusted incidence rate of GC is about 7.5 per 100,000 [2], whereas in Japan about 31 per 100,000 [3] suffer from GC. Ethnic differences in GC can be also found in the stage of GC at diagnosis, the sub-site distribution to cardia, fundus, body and pyloric antrum and treatment efficacy [4].

5-year survival rate in locally advanced GC is about 36% and cure can be achieved by an appropriate surgical approach with extended lymph-node resection (D2) combined with a perioperative chemo +/- radiotherapy [5, 6]. The surveillance procedures after removal of the primary tumor are critical for early detection and treatment of recurrence. The major pathways altered and accountable for progression and metastasis of GC are the Wnt/ β -catenin pathway [7], aurora-kinase [8] and epidermal growth factor receptor (ErbB) family driven pathways [9]. Erb-B2, also known as HER2, is overexpressed in about 20% of metastatic GC and the HER2 antibody trastuzumab is approved in the treatment of HER2 positive metastatic GC [10]. Although the EGF-receptor is upregulated in 30–60% of GC [11] treatment with antibodies directed against the epidermal growth factor receptor (EGFR) with cetuximab [12, 13] or panitumumab [14] have failed in metastatic disease when applied on an unselected patient population.

For patients with locally advanced GC it would be important to adjust their follow-up procedures to their individual risk of recurrence and tumor related death rate. EGFR signaling is important for metastasis and tumor progression and its prognostic value in GC was demonstrated in several recent studies [15–17]. *AREG* and *EREG* are the most important ligands for sustained signaling of the EGFR [18] but standardized tests to measure expression levels are difficult to establish and results depend on a variety of issues. Single nucleotide polymorphisms (SNPs) in contrast are easy to access offering a promising field for biomarker development.

This analysis was performed to establish the prognostic value of SNPs in *AREG* and *EREG* in patients with locally advanced gastric cancer for 3-year recurrence free and 3-year survival rate.

Methods

Patients

Two patient cohorts with histo-pathologically confirmed, localized adenocarcinoma of the stomach (GC) with AJCC stage IB-IV (6th version) were analyzed. Tumor material of 169 Japanese patients derived from patients treated at Fukushima Red Cross Hospital (n=118), Japan and patients treated within a study testing S-1 in the adjuvant setting of GC from Kitasato University East Hospital (n=51), Japan [5, 19]. Those patients were recruited between 1991 and 2011 and were treated with D2-based surgery alone, or D2-based surgery and adjuvant chemotherapy. A cohort of 137 gastric cancer patients treated at the Los Angeles County Hospital/University of Southern California Medical Center (n=105), or the Memorial Sloan-Kettering Cancer Center/Cornell University (n=32) was used as a validation set. Those patients were recruited between 1992 and 2008 and were treated with surgery alone, or surgery and adjuvant (radio)-chemotherapy [20]. All patients had given their written informed consent prior to entering the study. For detailed patient characteristics for both cohorts see table 1.

Methods

Formalin-fixed, paraffin embedded (FFPE) normal gastric tissues or venous blood samples were used to extract genomic DNA. As there is almost a complete concordance between blood and tumor tissue with regard to SNPs, the potential error by comparing SNPs from blood DNA and tissue DNA is neglectable [21]. DNA extraction was carried out using the QIAamp® DNA easy kit (Qiagen, Valencia, CA, USA) as recently published [22]. DNA was stored at -20°C until use. Common and potentially functional polymorphisms within the amphiregulin and epiregulin genes were selected by using the Ensembl database [23]. The following criteria were used to select the candidate gene polymorphisms: (a) a minor allele frequency (MAF) > 10%; (b) located in the 3'UTR, 5'UTR and coding regions of the tested genes and/or were shown to be of biological significance according to the location within the gene or according to literature.

The tested SNPs for *AREG* and *EREG* and their forward and reverse primer used for PCR amplification are shown in table 2.

PCR products were analyzed using direct sequencing. The investigator (TW) reading the sequence was blinded to the clinical results.

To correlate SNPs with gene-expression rtPCR was carried out, using β -actin as housekeeping mRNA. RNA extraction was done using a commercially available kit (QIAamp® RNAeasy kit (Qiagen, Valencia, CA, USA)).

Tumor cells were collected using laser-capture micro-dissection as described recently [24]. In short, three sections, each of 10-um thickness, were dewaxed, re-hydrated and stained

with nuclear fast red (American Master Tech Scientific, Lodi, CA, USA). Laser capture micro-dissection was done using an ArcturusVeritas 704 ® microdissection system (Molecular Devices, Sunnyvale, CA, USA).

Statistics

The primary endpoint of the current study was overall survival (OS) and secondary endpoint was recurrence free survival (RFS). As done by other studies dealing with post-surgery survival [25], 3-year recurrence rates and 3-year survival rates were calculated. OS was defined as the period from the date of surgery in the Japanese cohort and the date of diagnosis in the USC cohort to date of death from any cause, or censored on the last known date of being alive. RFS was calculated from the date of surgery (Japanese) or diagnosis (USC) to the first documented date of recurrence. RFS was censored on the date of last follow-up if patients remained recurrence free. The slight discrepancy (the starting point) in the definition of OS and RFS between the Japanese and USC cohorts was due to the difference in keeping and retrieving medical records. With the median follow-up of 4.0 years, 71 deaths and 80 recurrences were observed in the Japanese cohort. Among the USC cohort, 45 deaths and 61 recurrences were observed with a median follow-up of 3.3 years.

Allelic distribution of the *AREG* and *EREG* polymorphisms by race/ethnicity was tested for deviation from Hardy-Weinberg equilibrium using χ^2 test with one degree of freedom. To evaluate the prognostic value of the *AREG* and *EREG* polymorphisms on endpoints, the associations were examined using by Kaplan-Meier curve methods and tested by the log-rank test. The Cox proportional hazards regression model with covariates were fitted to re-evaluate the association between polymorphisms and outcomes considering the imbalanced in the distributions of baseline patient characters in both cohorts. Interactions between polymorphisms and gender or histo-logical tumor type on OS and RFS were tested by comparing likelihood ratio statistics between the baseline and nested Cox regression models that include the multiplicative product term.

With 169 patients in the Japanese cohort and 137 patients in the US cohort, we would have 80% power to detect a minimum hazard ratio of 1.96–2.80 and 2.33–3.39, respectively, in OS across a range of the variant allele frequencies (0.05–0.4) in a dominant model using a 0.05 level two-sided log-rank test. For a recessive model, the minimum hazard ratio is about 3.47 and 4.29 in the Japanese and USC cohorts, respectively, when the variant allele frequency is 0.25 and approaches 2.09 and 2.45, respectively, when the allele frequency is 0.5.

The level of significance was set to 0.05, and all statistical tests were two-sided and performed using the SAS statistical package version 9.3 (SAS Institute, Cary, NC, USA).

Results

The primary outcomes of both trials have been reported recently [5, 16, 19]. Table 1 summarizes baseline patient characteristics. Direct sequencing after PCR was successful in more than 98% of the samples. For quality control purposes a random selection of 10% of the samples was re-examined for each polymorphisms and genotype concordance rate was

100%. The allele frequencies of *AREG* rs1615111 and *EREG* rs12641042 were as expected [26] and comparable between both cohorts (table 3). *AREG* rs1615111 minor allele frequency (adenine) was 6.8% in Japanese and 6.4% in US-American cohort. For *EREG* rs12641042, minor allele frequencies (adenine) were 39.6% and 38.9% respectively. No linkage disequilibrium could be established between *AREG* rs1615111 and *EREG* rs12641042.

All patients were without detectable tumor after surgery. The results of uni- and multivariable testing adjusted for age, gender, and stage, are given in table 4. Univariable analysis using Kaplan-Meier estimation and log-rank testing revealed for the G/A genotype of *AREG* rs1615111 a higher 3-year recurrence rate of 71% (standard error (SE): $\pm 10\%$), compared to 44% (SE: $\pm 4\%$) in patients having the GG genotype (HR: 1.98 (95% confidence interval (CI): 1.14 – 3.44, log-rank test $p = 0.012$). This translated into a lower 3-year survival rate for the minor allele A of 40% (SE: $\pm 11\%$) when compared to the homozygous GG (66%; SE: $\pm 4\%$) (HR 1.83, log-rank test $p = 0.039$). This negative value was conserved when multivariable analyses was applied with a HR of 2.21 (95% CI: 1.24 – 3.94; Wald test $p = 0.007$) for 3-year recurrence rate and a HR of 2.35 (95% CI: 1.26 – 4.36; Wald test $p = 0.007$). In univariable testing, the major allele C of *EREG* rs12641042 was associated with a significantly lower 3-year survival rate of 58% (SE: $\pm 4\%$) when compared to the homozygous AA genotype (80%; SE: $\pm 7\%$, HR: 0.48; 95% CI 0.24 – 0.96, log-rank test $p = 0.034$). This value was lost when multivariable testing was applied (Wald test $p = 0.066$). *EREG* rs12641042 within the cohort of Japanese patients was not within the Hardy-Weinberg equilibrium ($p = 0.013$), despite of repeated direct sequencing. All other SNPs tested for their predictive value were not able to separate different prognostic groups within this cohort of Japanese gastric cancer patients (table 4a).

In a second step, the values of *AREG* rs1615111 and *EREG* rs12641042 were tested in the US patient cohort (table 4b). 3-year recurrence rate was higher for patients bearing the minor allele A of *AREG* rs1615111 (74%, SE: $\pm 15\%$) when compared to the homozygous GG cohort (50%, SE: $\pm 6\%$) but this difference did not reach level of significance (HR: 1.53, 95% CI: 0.69 – 3.41, log-rank test $p = 0.29$). When *AREG* rs1615111 was tested for 3-year survival rate, its value could be validated. Patients bearing the minor allele A had a significantly lower 3-year survival rate of 35% (SE: $\pm 16\%$) when compared to patients having the homozygous genotype GG (76%, SE: $\pm 5\%$; HR: 2.54, 95% CI: 1.09 – 5.89, log-rank test $p = 0.018$) (figure 1).

EREG rs12641042 could not be validated as a predictor for 3-year survival rate (67% vs. 70%, HR= 1.01, 95% CI: 0.39 – 2.63, log-rank test $p = 0.98$) in the US cohort (figure 1). The 3-year recurrence rate was 36% (SE: $\pm 20\%$) for patients homozygous for the minor allele A and 61% (SE: $\pm 7\%$) for patients bearing the major allele G but this difference did not reach level of significance (HR: 0.56, 95% CI: 0.22 – 1.41; log-rank test $p = 0.20$).

To investigate the influence of gender and tumor type (intestinal/diffuse) time-to recurrence and overall survival for *AREG* rs1615111 and *EREG* rs12641042 an exploratory subgroup analysis was carried out combining both patient cohorts (figure 2). For both SNPs the prognostic value was limited to male patients. *AREG* rs1615111 was further of special

predictive value with-in the group of diffuse tumors ($P_{\text{for interaction}} = 0.004$) whereas *EREG* rs12641042 had a trend of being more decisive for intestinal tumor types.

Furthermore we tested the influence of the significant SNP *AREG* rs1615111 on *AREG* gene-expression. Due to the samples coming from pretreatment biopsies we were able to obtain mRNA in adequate quality and quantity in only 85 (50.3%) samples of Japanese gastric cancer. Twelve had “AG” genotype and 73 were “GG”. There was no difference in *AREG* gene-expression (two sided t-test $p = 0.52$) with a median of 0.39 (95% CI: 0.26 – 0.66) for the AG genotype and 0.35 (95% CI: 0.24 – 0.59) for the homozygous GG samples.

Discussion

In this study SNP rs1615111 in *AREG* was able to define subpopulations of GC patients with significantly different 3-year survival rates after surgery with curative intent. This value was validated in a second, independent population and was most prominent in male patients or patients with a diffuse histology of GC.

In GC adjuvant and neoadjuvant therapies improve outcome and the combination of surgery, chemotherapy, and radiation offers cure for a fraction of patients [6, 27]. Due to different etiology, tumor location, and ethnicity, perioperative treatment procedures differ between Japan and the US. In Japan S1 has shown efficacy [28] and is widely used whereas in the US a combination of chemotherapy and radiation is regarded as standard [29]. However, D2 lymphnode resection is the accepted gold-standard surgical procedure to prolong survival [30]. Perioperative therapy and D2 resection especially for older patients are associated with a markedly high morbidity [31]. Therefore, biomarkers defining a subpopulation of locally advanced gastric cancer patients with a higher risk of cancer recurrence and death would be able to guide clinical decision making and further personalize GC treatment. With the exception of HER2-expression in the treatment of metastatic GC, no biomarker has been established in GC so far [32].

For cancer recurrence after surgery with curative intent, pathways modulating cell-stemness are of most interest. Recent in vitro studies have revealed a critical role of EGFR signaling for stemness in GC cell lines [33]. This role for EGFR signaling is supported by the observation that EGF-receptor expression and the expression levels of the EGFR ligands *AREG* and *EREG* are also associated with prognosis and relapse prediction [15]. But expression analysis in GC with only biopsy material at the time of diagnosis is limited. Methodological issues for expression analysis such as cut-off definition, definition of housekeeping proteins and single cell laser microdissection are either not defined or impractical for everyday clinical practice. Therefore SNPs which are easy to access would be ideal as biomarkers. The current analysis focused for the first time in gastric cancer on SNPs within genomic regions of *AREG* and *EREG* and their association with 3-year recurrence free (RFS) and 3-year overall survival rates.

AREG rs161511 in the intergenic region of amphiregulin was able to define a fraction of 11–13% of patients with locally advanced GC that have a higher probability of recurrence and early death. The difference in 3-year recurrence rate was significant in the Japanese cohort

but did not reach level of significance in the US-American cohort. Reasons to explain those differences between both cohorts are, among others, the extent of the surgical procedure and the biology and aggressiveness of the tumor, reflected by the histology and the location of the primary tumor (see table 1).

In the subgroup of patients where sufficient RNA expression could be measured, mRNA-expression of *AREG* was not dependent on the rs1615111 genotype. This might be due to the limited number of samples (50.3%) or due to the fact that the functional difference might be to the post-transcriptional influence of rs1615111. But protein expression has not been tested. Data from the RegulomeDB database [34] indicate that rs1615111 is not changing the respective protein (synonymous SNP), but is associated with a transcription factor binding site and a DNase peak, which both should lead to modulation in transcriptional regulation. The functional meaning of rs1615111 therefore has to be further investigated.

In silico data for rs12641042 is limited and with only minimal binding evidence reported, function on transcriptional regulation is minor [34]. Using the dominant model, rs12641042 was able to define a subgroup of about 20% of patients that had a longer OS in the Japanese cohort and a longer recurrence-free survival (RFS) in the US-American cohort. The value to predict OS could therefore not been validated. Next to the fact that it may be a false positive finding in the Japanese cohort, this might be due to ethnicity or the discrepancies of surgical procedure and peri-operative chemotherapy between the training and the validation set.

The association of gender and tumor type was analyzed for both SNPs rs1615111 and rs12641042. Notably, both SNPs prognostic associations were only seen in male patients, suggesting the EGFR pathway activation is of special importance for male patients. This may be one explanation for the gender related differences seen in GC survival [35]. Furthermore estrogen signaling increases the PI3KCA/Akt-mediated metastasis in GC [36] and therefore may be able to bypass EGFR dependent signaling.

The separation of rs1615111 was most significant in patients with a diffuse tumor type in both, the Japanese and the US-American cohort, indicating a specific role for EGFR signaling in diffuse GC. This is in line with observations from the EXPAND study where patients with a diffuse tumor type did significantly worse when treated with cetuximab in metastatic GC [12].

Gastric cancer obviously is a heterogeneous disease and more detailed analyses of the activated/dysregulated pathways might lead to biomarkers separating different GC types and further define subgroups that might qualify for EGFR-inhibitor treatment.

Even though the current analysis was able to validate the value of *AREG* SNP rs161511 to predict 3-year survival rates in GC patients after surgery in curative intent, this study has several limitations. Among those are the retrospective character of the analysis as far as the differences between the training and the validation population in terms of tumor extent, ethnicity and treatment. In this context, the ethnical heterogeneity of the US cohort has to be mentioned. In multivariable analysis, the numbers of female patients are small so

conclusions should be drawn carefully. Furthermore this analysis is rather hypothesis generating and results should be replicated in other cohorts.

Conclusion

This is the first report of SNPs in genetic association with the EGFR ligands AREG and EREG as biomarker in locally advanced GC. *AREG* rs1615111 showed a significant prognostic value for patients with GC after surgery with curative intent in two independent and ethnically different cohorts. Its prognostic value varies with regard to gender and tumor type, reflecting the heterogeneity GC patients and the pathways activated in carcinogenesis and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the NCI CCSG grant 5P30CA014089-27S1 and the Nancy Bernstein Research Fund. S. Stintzing is currently receiving a postdoctoral fellowship by the German Cancer Aid (Mildred-Scheel Foundation).

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Figure 1. Kaplan-Meier-estimation curves of AREG rs161511 and EREG rs12641042 for median OS times and 3-year survival rates

yrs = years, nr = not reached, HR = hazard ratio; CI = confidence interval; A = adenine, G = guanine, n = number of patients, p^* = logrank test p (univariate),

Figure 2. Forest plot: Association of gender and tumor differentiation on outcome data of AREG rs1615111 (A) and EREG rs12641042 (B) taken both cohorts (US and Japanese) together
RFS = recurrence free survival; OS = overall survival, HR = Hazard ratio, A = adenine, G = guanine, T = thymine, C = cytosine, * = significant association, p-value = Wald test p; n = number of patients (any A/GG and AA/any C respectively)

Table 1

Baseline characteristics

Characteristic	Japanese Cohort N= 169	US Cohort N= 137
Age,		
median years (range)	67 (31 – 88)	57 (21 – 85)
>65 years, %	36	23
>75 years, %	26	9
Gender		
male, %	64	61
female, %	36	39
ECOG – PS		
0, %	93	45
1, %	7	55
AJCC Stage		
IB, %	7	9
II, %	39	26
III, %	52	52
IV, %	2	13
N-Status		
N0, %	21	20
N1, %	50	47
N2, %	20	23
N3, %	9	7
Tumor site		
Stomach, %	96	64
GEJ, %	2	28
unknown, %	2	8
Tumor type		
Intestinal / mixed, %	40	64
Diffuse, %	60	36
Chemotherapy, %	64	82
Ethnicity		
Japanese	100	
Caucasian		46
Hispanic		33
African American		1
Asian		20

N = number of patients, ECOG-PS = Eastern Cooperative Oncology Group performance status; AJCC: American Joint committee on cancer; N-Status: Lymph node status according to the TNM classification of malignant tumors of the AJCC; GEJ = tumor of the gastro-esophageal junction

Table 2

Analyzed polymorphisms within AREG and EREG

Gene SNP	Allele	MAF	Function	Forward (F) and Reverse (R) primer
AREG rs161511	G>A	7%	splicing regulation	F: 5'-TCTCCACTCGCTCTTCCAAC-3' R: 5'-CCTCACCTGAGCCGAGTATC-3'
AREG rs28364983	T>C	23%	transcriptional regulation	F: 5'-ATGGCTGTTACCCCT GTGAG-3' R: 5'-GGGGGCTTAACCTACCTGTTCA-3'
AREG rs1691263	C>T	25%	unknown	F: 5'-AAAAAGGGAGGCAAAAATGG-3' R: 5'-CCTGCAGTTAGGGGTTTCAG-3'
AREG rs2291715	A>C	32%	splicing regulation, transcriptional regulation	F: 5'-AAAACACACCGCACGTTTTT-3' R: 5'-AACAGCAACAGCTGTGAGGA-3'
EREG rs10446508	A>T	36%	transcriptional regulation	F: 5'-CCCCAAAACCTCTTCTACCTTT-3' R: 5'-CAGCCCATGGAAATATAGGG-3'
EREG rs1962685	C>T	25%	transcriptional regulation	F: 5'-CTCCAAAACCTGCTGGGATTA-3' R: 5'-GGGCACAGATGTTCAAGTCA-3'
EREG rs2061509	C>T	31%	transcriptional regulation	F: 5'-TCCTTAGAAGGGAGGAAGGAA-3' R: 5'-GGGATGAAGAGGTGATGTGC-3'
EREG rs7687621	C>T	17%	transcriptional regulation	F: 5'-TGATGTGGTTTCCTCAAAGC-3' R: 5'-ATAATGTGCCAAGGTCCAA-3'
EREG rs12641042	C>A	46%	transcriptional regulation	F: 5'-TCCAGTCAGAAACCATGCAA-3' R: 5'-CACAGTGCAAGTCTGCTGGTA-3'
EREG rs7575690	T>C	15%	transcriptional regulation	F: 5'-CTGCATGATTTCCAGGATTG-3' R: 5'-TGCTACCTCCAAATTCAACA-3'

AREG = amphiregulin; EREG = epieregulin, MAF = minor allele frequency (Japanese ethnicity), T= thiamine, C= cysteine, A= adenine, G= guanine

Table 3

Frequency of the analyzed polymorphisms within AREG and EREG

Gene SNP	Allele	MAF Literature Japanese*	MAF Japanese Cohort	MAF Literature European*	MAF US Cohort
AREG rs161511	G>A	9%	6.8%	7%	6.4%
AREG rs28364983	T>C	23%	26.0%		na
AREG rs1691263	C>T	25%	14.6%		na
AREG rs2291715	A>C	32%	20.4%		na
EREG rs10446508	A>T	36%	36.8%		na
EREG rs1962685	C>T	25%	21.3%		na
EREG rs2061509	C>T	31%	26.8%		na
EREG rs7687621	C>T	17%	25.0%		na
EREG rs12641042	C>A	46%	39.6%	27%	38.9%
EREG rs7575690	T>C	15%	10.5%		na

AREG = amphiregulin; EREG = epi-regulin, MAF = minor allele frequency, T= thiamine, C= cysteine, A= adenine, G= guanine, *= Ensembl database

Table 4a

Association of the selected SNPs with endpoints in Japanese gastric cancer patients

		Recurrence free survival			Overall survival		
SNP	N	3-year recurrence rate ± SE	HR [#] (95% CI)	HR [‡] (95% CI)	3-year survival rate ± SE	HR [#] (95% CI)	HR [‡] (95% CI)
AREG rs1615111							
G/G	146	0.44 ±0.04	reference	reference	0.66 ±0.04	reference	reference
G/A	23	0.71 ±0.10	1.98 (1.14, 3.44)	2.21 (1.24 – 3.94)	0.40 ±0.11	1.83 (1.02 – 3.30)	2.35 (1.26 – 4.36)
	P*		0.012	0.007		0.039	0.007
AREG rs28364983							
T/T	94	0.45 ±0.05	reference	reference	0.67 ±0.05	reference	reference
T/C	62	0.50 ±0.07	1.16 (0.73 – 1.84)	1.13 (0.7 – 1.81)	0.56 ±0.07	1.18 (0.72 – 1.93)	1.18 (0.7 – 1.98)
C/C	13	0.46 ±0.14	1.07 (0.45 – 2.51)	1.27 (0.52 – 3.07)	0.53 ±0.14	1.36 (0.57 – 3.23)	1.44 (0.59 – 3.53)
	P*		0.82	0.81		0.69	0.66
AREG rs1691263							
C/C	136	0.48 ±0.04	reference	reference	0.61 ±0.04	reference	reference
C/T	15	0.33 ±0.12	0.68 (0.27 – 1.68)	0.43 (0.17 – 1.09)	0.80 ±0.10	0.48 (0.15 – 1.53)	0.3 (0.09 – 0.97)
T/T	17	0.51 ±0.13	1.01 (0.48 – 2.11)	1.35 (0.62 – 2.95)	0.67 ±0.12	0.74 (0.32 – 1.71)	0.72 (0.3 – 1.73)
	P*		0.69	0.15		0.37	0.11
AREG rs2291715							
A/A	108	0.49 ±0.05	reference	reference	0.64 ±0.05	reference	reference
A/C	53	0.44 ±0.07	0.88 (0.55 – 1.39)	1.01 (0.61 – 1.67)	0.59 ±0.07	1.01 (0.62 – 1.64)	1.05 (0.62–1.73)
C/C	8						
	P*		0.58	0.98		0.97	0.86
EREG rs10446508							
A/A	74	0.48 ±0.06	reference	reference	0.63 ±0.06	reference	reference
A/T	63	0.41 ±0.06	0.93 (0.56 – 1.54)	0.99 (0.58 – 1.69)	0.67 ±0.06	0.87 (0.51 – 1.50)	0.90 (0.51 – 1.60)
T/T	30	0.59 ±0.09	1.21 (0.68 – 2.17)	1.06 (0.59 – 1.91)	0.52 ±0.10	1.20 (0.65 – 2.22)	1.12 (0.60 – 2.09)
	p*		0.69	0.97		0.63	0.83
EREG rs1962685							

SNP	N	Recurrence free survival			Overall survival		
		3-year recurrence rate $\pm$ SE	HR [#] (95% CI)	HR [‡] (95% CI)	3-year survival rate $\pm$ SE	HR [#] (95% CI)	HR [‡] (95% CI)
C/C	110	0.51 $\pm$ 0.05	reference	reference	0.60 $\pm$ 0.05	reference	reference
C/T	46	0.40 $\pm$ 0.07	0.81 (0.48 – 1.37)	1.13 (0.65 – 1.96)	0.63 $\pm$ 0.07	0.85 (0.49 – 1.47)	1.13 (0.62 – 2.03)
T/T	13	0.38 $\pm$ 0.13	0.70 (0.28 – 1.74)	0.80 (0.32 – 2.03)	0.77 $\pm$ 0.12	0.79 (0.31 – 1.98)	0.99 (0.39 – 2.54)
	<i>P</i> *		0.58	0.80		0.77	0.92
EREG rs2061509							
C/C	96	0.52 $\pm$ 0.05	reference	reference	0.58 $\pm$ 0.05	reference	reference
C/T	54	0.38 $\pm$ 0.07	0.72 (0.44 – 1.20)	1.03 (0.60 – 1.76)	0.71 $\pm$ 0.06	0.67 (0.39 – 1.16)	0.91 (0.51 – 1.62)
T/T	18	0.45 $\pm$ 0.12	0.81 (0.38 – 1.70)	0.93 (0.44 – 1.98)	0.66 $\pm$ 0.11	0.92 (0.43 – 1.95)	1.18 (0.54 – 2.54)
	<i>P</i> *		0.43	0.97		0.36	0.84
EREG rs7687621							
C/C	99	0.52 $\pm$ 0.05	reference	reference	0.59 $\pm$ 0.05	reference	reference
C/T	51	0.38 $\pm$ 0.07	0.75 (0.45 – 1.25)	1.03 (0.60 – 1.77)	0.71 $\pm$ 0.07	0.69 (0.40 – 1.21)	0.88 (0.49 – 1.59)
T/T	16	0.38 $\pm$ 0.12	0.67 (0.29 – 1.56)	0.78 (0.33 – 1.83)	0.68 $\pm$ 0.12	0.78 (0.33 – 1.84)	0.99 (0.42 – 2.38)
	<i>P</i> *		0.41	0.83		0.41	0.91
EREG rs12641042							
C/C	69	0.53 $\pm$ 0.06	reference	reference	0.58 $\pm$ 0.06	reference	reference
C/A	65	0.48 $\pm$ 0.06	0.97 (0.60 – 1.57)	0.92 (0.56 – 1.50)	0.58 $\pm$ 0.06	1.07 (0.64 – 1.76)	1.11 (0.67 – 1.85)
A/A	34	0.33 $\pm$ 0.08	0.58 (0.30 – 1.12)	0.57 (0.29 – 1.11)	0.80 $\pm$ 0.07	0.49 (0.23 – 1.04)	0.53 (0.25 – 1.15)
	<i>P</i> *		0.23	0.25		0.10	0.17
C/C or C/A	134	0.50 $\pm$ 0.04	reference	reference	0.58 $\pm$ 0.04	reference	reference
A/A	34	0.33 $\pm$ 0.08	0.59 (0.32 – 1.09)	0.59 (0.31 – 1.11)	0.80 $\pm$ 0.07	0.48 (0.24 – 0.96)	0.51 (0.25 – 1.05)
	<i>P</i> *		0.088	0.10		0.034	0.066
EREG rs7575690							
T/T	135	0.44 $\pm$ 0.04	reference	reference	0.66 $\pm$ 0.04	reference	reference
T/C	29	0.63 $\pm$ 0.09	1.52 (0.90 – 2.55)	1.22 (0.72 – 2.07)	0.49 $\pm$ 0.09	1.56 (0.90 – 2.70)	1.27 (0.73 – 2.22)
C/C	3						
	<i>P</i> *		0.11	0.45		0.11	0.40

SNP = single nucleotide polymorphisms, HR = hazard ratio; $\pm$ SE = rate $\pm$ standard error, CI = confidence interval; AREG = amphiregulin, EREG = epiregulin; A = adenine, G = guanine, T = thymine, C = cytosine, N= number of patients;

* *P* (statistical significant value bold typed) was based on log-rank test for RFS and OS in the univariable analysis (#) and Wald test for RFS and OS in the multivariable Cox regression model ([‡]).

[‡] Adjusting for sex (male vs. female), stage (I, II, III, and IV as categorical), age (<65, 65–74, $\geq$ 75+ years as ordinal), and type of adjuvant therapy (no vs. yes).

Table 4b

Association of the selected SNPs with endpoints in US gastric cancer patients

		Recurrence free survival			Overall survival		
SNP	N	3-year recurrence rate ± SE	HR [#] (95% CI)	HR [‡] (95% CI)	3-year survival rate ± SE	HR [#] (95% CI)	HR [‡] (95% CI)
AREG rs161511							
G/G	111	0.50 ±0.06	reference	reference	0.76 ±0.05	reference	reference
G/A [§]	12	0.74 ±0.15	1.53 (0.69, 3.41)	0.99 (0.34, 2.86)	0.35 ±0.16	2.54 (1.09, 5.89)	3.15 (0.96, 10.31)
A/A [§]	2						
	<i>P</i> [*]		0.29	0.98		0.018	0.058
EREG rs12641042							
C/C	41	0.71 ±0.09	reference	reference	0.64 ±0.09	reference	reference
C/A	45	0.49 ±0.09	0.61 (0.33, 1.14)	0.45 (0.20, 1.00)	0.70 ±0.09	0.54 (0.26, 1.10)	0.56 (0.23, 1.37)
A/A	18	0.36 ±0.20	0.44 (0.17, 1.15)	0.19 (0.05, 0.68)	0.70 ±0.18	0.77 (0.29, 2.06)	0.52 (0.16, 1.75)
	<i>P</i> [*]		0.11	0.020		0.22	0.35
C/C or C/A	86	0.61 ±0.07	reference	reference	0.67 ±0.06	reference	reference
A/A	18	0.36 ±0.20	0.56 (0.22, 1.41)	0.28 (0.08, 0.96)	0.70 ±0.18	1.01 (0.39, 2.63)	0.68 (0.22, 2.12)
	<i>P</i> [*]		0.20	0.043		0.98	0.50

* *P* was based on log-rank test for TTR and OS in the univariable analysis (#) and Wald test for TTR and OS in the multivariable Cox regression model (§).

[‡] Adjusted for tumor stage (T1-2 vs. T3-T4), N stage (N0, N1 vs. N2, N3) and stratified by race (four groups: White, African American, Asian, and Hispanic) and adjuvant therapy (four groups: 5-FU/LV; 5-FU/LV/oxaliplatin; 5-FU, CDDP, CPT-11; none).

[§] Combined in the analysis.