

Epidermal growth factor receptor intron 1 CA dinucleotide repeat polymorphism and survival of advanced gastric cancer patients treated with cetuximab plus modified FOLFOX6

Sae-Won Han,^{1,2} Do-Youn Oh,^{1,2} Seock-Ah Im,^{1,2} Sook Ryun Park,³ Keun-Wook Lee,⁴ Hong Suk Song,⁵ Nam-Su Lee,⁶ Kyung Hee Lee,⁷ In Sil Choi,⁸ Moon Hee Lee,⁹ Min A Kim,¹⁰ Woo Ho Kim,¹⁰ Yung-Jue Bang^{1,2,11} and Tae-You Kim^{1,2,11}

¹Department of Internal Medicine, Seoul National University Hospital, Seoul; ²Cancer Research Institute, Seoul National University College of Medicine, Seoul; ³National Cancer Center, Goyang; ⁴Seoul National University Bundang Hospital, Seongnam; ⁵Keimyung University School of Medicine, Daegu; ⁶Soonchunhyang University College of Medicine, Seoul; ⁷Yeungnam University College of Medicine, Daegu; ⁸Seoul Municipal Boramae Hospital, Seoul; ⁹Inha University Hospital, Incheon; ¹⁰Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

(Received September 6, 2009/Revised November 8, 2009/Accepted November 14, 2009/Online publication December 25, 2009)

Cetuximab is a monoclonal antibody targeting epidermal growth factor receptor (EGFR). The present study investigated the association between germline genetic polymorphisms and the treatment outcome of cetuximab plus modified leucovorin, fluorouracil, and oxaliplatin (FOLFOX)6 chemotherapy in advanced gastric cancer (AGC). DNA from peripheral blood mononuclear cells of 38 patients enrolled in a phase II study of cetuximab plus modified FOLFOX6 were analyzed for 16 polymorphisms in eight genes (EGFR, epidermal growth factor, transforming growth factor- α (TGFA), thymidylate synthase, excision repair cross-complementation group 1, *Xeroderma pigmentosum* group D, and fragment c gamma receptors (FCGR)2A and 3A). The EGFR intron 1 CA repeat polymorphism was associated with survival. Twenty-one patients had low repeats (sum of both alleles ≤ 37), and 17 patients had high repeats (sum ≥ 38). Patients with low CA repeats had longer progression-free survival (adjusted hazard ratio [HR] 0.42 [95% confidence interval [CI] 0.19–0.96], $P = 0.040$) and overall survival (adjusted HR 0.40 [95% CI 0.16–0.99], $P = 0.048$) compared with patients with high CA repeats. In addition, the tumor EGFR expression was higher in patients with a lower number of CA repeats. The association between the CA repeat status and survival was not found in a separate cohort of AGC patients ($n = 68$) treated only with modified FOLFOX6. These results suggest that the EGFR intron 1 CA repeat polymorphism could be a useful, predictive biomarker of cetuximab efficacy in AGC and merits further investigation in randomized studies. (*Cancer Sci* 2010; 101: 793–799)

Gastric cancer is frequently associated with poor survival because it often presents as unresectable disease, and chemotherapy shows limited efficacy.⁽¹⁾ Therefore, gastric cancer is a major health concern in many countries, including Korea, which has a particularly high incidence.^(1,2) In order to improve the treatment outcome of chemotherapy in advanced gastric cancer (AGC), targeted agents are being actively investigated.⁽³⁾ Recently, trastuzumab, a monoclonal antibody targeting human epidermal growth factor receptor 2 (HER2), in addition to fluoropyrimidine and cisplatin, significantly improved the overall survival in HER2-positive gastric cancer in a phase III study.⁽⁴⁾

Cetuximab (Erbix; Merck KGaA, Darmstadt, Germany) is a monoclonal antibody that binds to and inactivates epidermal growth factor receptor (EGFR).⁽⁵⁾ Cetuximab improved the treatment outcome of metastatic colorectal cancer patients.^(6,7) Interestingly, the benefit of cetuximab was limited to K-ras wild-type colorectal cancers.^(7,8) These and other similar findings led to the

recommendation that metastatic colorectal patients with K-ras mutant tumors should not receive anti-EGFR therapy.⁽⁹⁾

Cetuximab plus chemotherapy has also shown favorable results as a first-line treatment of advanced gastric or gastroesophageal junction adenocarcinoma in phase II studies.^(10,11) Based on these results, a phase III study to evaluate the benefit of cetuximab in addition to capecitabine and cisplatin in advanced esophagogastric cancer is currently underway.⁽¹²⁾ In contrast to colorectal cancer, K-ras mutation is infrequently found in gastric cancer.⁽¹³⁾ Therefore, other predictive biomarkers should be investigated to aid patient selection for cetuximab in gastric cancer.

We have also conducted a phase II study of cetuximab in AGC.⁽¹⁴⁾ Although cetuximab in combination with modified leucovorin fluorouracil and oxaliplatin (FOLFOX)6 failed to meet the prespecified improvement in the response rate, patients with a tumor EGFR expression and low serum ligand levels showed favorable outcomes in the exploratory biomarker analysis.⁽¹⁴⁾ In the present study, we investigated candidate genetic polymorphisms and their association with the treatment outcome.

Materials and Methods

Patients and treatment. Patients who were enrolled in the Korean Cancer Study Group prospective multicenter phase II study of cetuximab in combination with modified FOLFOX6 were included in the present analysis. The main inclusion criteria of the study were age ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 2 ; histologically-confirmed adenocarcinoma of the stomach; recurrent or metastatic disease, no prior chemotherapy, radiotherapy, immunotherapy, or EGFR pathway-targeting therapy; adequate bone marrow, hepatic, and renal function; and at least one measurable lesion. Patients received an initial dose of 400 mg/m² cetuximab, followed by weekly doses of 250 mg/m². Modified FOLFOX6 was comprised of 100 mg/m² oxaliplatin and 100 mg/m² leucovorin administered intravenously over 2 h on day 1, followed by a 46-h infusion of 2400 mg/m² 5-fluorouracil (5-FU), which was repeated every 2 weeks. Patients received a maximum of 12 cycles of modified (m) FOLFOX6. Cetuximab was continued as a monotherapy until disease progression. A response evaluation was performed following the RECIST criteria.⁽¹⁵⁾ Detailed results of the efficacy and toxicity have been

¹¹To whom correspondence should be addressed.
E-mail: kimty@snu.ac.kr

reported previously.⁽¹⁴⁾ Among the 40 patients enrolled in the phase II study, the study included 38 patients, excluding two patients whose responses were not evaluable (Table 1). In addition, two patients with unconfirmed partial response (PR) were considered to be responders in the present study. Survival data were last updated in May 2009. Another group of AGC patients in a phase II study of modified FOLFOX6 were also analyzed for the EGFR polymorphism.⁽¹⁶⁾ Among the 73 patients enrolled in the original study, 68 patients were included in the present study because five patients had no remaining DNA sample (Table S1). All patients, including the patients who were enrolled in the mFOLFOX6 study,⁽¹⁶⁾ gave written, informed consent prior to study entry for the clinical study and biomarker analysis. The study protocol was reviewed and approved by the Institutional Review Boards at the participating institutions. Recommendations of the Declaration of Helsinki for biomedical research involving human participants were also followed.

Genotype analysis. For the analysis of germline genetic polymorphisms, genomic DNA was extracted from pretreatment peripheral blood samples using the QIAmp DNA blood kit (Qiagen, Valencia, CA, USA). Sixteen polymorphisms in eight genes were investigated. The following polymorphisms were analyzed using polymerase chain reaction–restriction fragment length polymorphism methods: epidermal growth factor (EGF; G61A), TGFA (*TaqI*, *RsaI*, *BamHI*), thymidylate synthase (TS; 28-bp repeat in the enhancer region, G/C polymorphism in the second repeat, 6-bp deletion in the 3'-untranslated region [UTR]), excision repair cross-complementation group 1 (ERCC1; Asn118Asn, C8092A), *Xeroderma pigmentosum* group D (XPD; Arg156Arg, Asp312Asn), and fragment c gamma receptor (FCGR)2A (His166Arg). Polymorphisms in transforming growth factor α (TGFA) (C3296T, C3827T) and FCGR3A (Val212Phe) were analyzed by direct sequencing. Primer sequences and restriction enzymes are listed in Table 2. The CA dinucleotide simple sequence repeat (SSR) polymorphism in intron 1 of EGFR was analyzed with a fragment length analysis using fluorescently-labeled primers, as described previously.⁽¹⁷⁾ All genotypes were in the Hardy–Weinberg equilibrium (data not shown).

Table 1. Baseline characteristics

Characteristic	No. patients (n = 38)	%
Sex		
Male	28	73.7
Female	10	26.3
Age, years		
Median	56.5	–
Range	41–74	–
Performance status (ECOG)		
0	7	18.4
1	28	73.7
2	3	7.9
Lauren classification		
Intestinal	12	31.6
Diffuse	26	68.4
No. organs involved		
1	5	13.2
2	7	18.4
≥ 3	26	68.4
Site of metastasis		
Lymph node	34	89.5
Peritoneum	19	50.0
Liver	15	39.5
Others (lung, bone etc.)	9	23.7

ECOG, Eastern Cooperative Oncology Group.

Statistical analysis. The statistical analysis of genetic status, baseline characteristics, and response rate was carried out using Pearson's chi-squared test, Fisher's exact test, or linear-by-linear association test where appropriate. The correlation between the CA repeat number and tissue EGFR expression was examined using the Mann–Whitney *U*-test or Spearman's rank correlation. Serum ligand levels were compared using the Mann–Whitney *U*-test or Kruskal–Wallis test. Median durations of progression-free-survival (PFS) and overall survival (OS) were calculated using the Kaplan–Meier method. Unadjusted comparisons of PFS and OS were made with log-rank tests. A multivariate analysis of response was performed with the backward stepwise logistic regression model. The multivariate analysis of PFS and OS was carried out using the backward stepwise Cox regression model. The following covariates were included to adjust for baseline characteristics: sex, age (older vs younger than median), ECOG PS (0 vs 1–2), Lauren classification, and additional characteristics with $P < 0.20$ (peritoneal seeding [response, PFS, OS], liver metastasis [OS], and number of organs involved [1–2 vs ≥ 3 , OS]). Same covariates were included in the multivariate analysis of the mFOLFOX6-only patient cohort, but the ECOG PS was categorized as 1 versus 2 because no patient had a PS of zero in the study. In the backward stepwise model, the covariate selection was performed using likelihood ratio statistics based on the conditional parameter estimate. The criteria for entry and removal were 0.05 and 0.10, respectively. Two-sided P -values of < 0.05 were considered significant. All analyses were performed using SPSS for Windows, version 12.0 (SPSS, Chicago, IL, USA).

Results

EGFR CA–SSR polymorphism. The most frequent genotype of the EGFR intron 1 CA–SSR polymorphism was 16/20 repeats found in 11 patients, followed by 20/20 repeats in 10 patients. Repeat lengths in the remaining patients were 19/20 in four patients, 16/16, 15/20, and 15/16 in two patients each, and 20/22, 20/21, 18/20, 16/17, 15/19, 14/20, and 14/16 in one patient each. For the statistical analysis, we classified patients as having either low or high CA repeats, according to the sum of repeat numbers in both alleles based on our previous study in non-small cell lung cancer.⁽¹⁷⁾ Twenty-one patients had low repeats (sum ≤ 37), and 17 patients had high repeats (sum ≥ 38).

The tumor EGFR expression, determined by the immunohistochemistry score (intensity \times percentage of positive cells), was higher in patients with low CA repeats compared to patients with high CA repeats ($P = 0.011$ by Mann–Whitney *U*-test). We analyzed the association between the CA repeat length and EGFR expression in patients with at least one 20 repeat allele ($n = 31$), which is the most frequent allele, to see how the CA repeat number in a single allele affects the EGFR expression. Patients with longer CA repeats in the remaining allele tended to have a lower EGFR expression (Spearman's $\rho = -0.46$, $\rho = 0.010$) (Fig. 1). No correlation was found between the CA repeat length and serum EGFR level (Spearman's $\rho = -0.060$, $P = 0.75$).

The CA repeat status (low vs high) was not associated with baseline characteristics. Although no significant association was found between the CA repeat status and response, PFS, or OS in the unadjusted analysis (Table 3), there were significant associations between CA repeat status and PFS and OS after adjusting for baseline characteristics. Patients with low CA repeats had longer PFS (adjusted HR 0.42 [95% confidence interval [CI] 0.19–0.96], $P = 0.040$) and OS (adjusted HR 0.40 [95% CI 0.16–0.99], $P = 0.048$) compared to patients with high CA repeats (Fig. 2A; Table 4). In addition, patients with low CA repeats were more likely to develop skin rashes (\geq grade 2) compared with high-repeat patients (66.7% vs 35.3%, respectively; $P = 0.054$).

Table 2. Primer sequences and restriction enzymes

Gene	Polymorphisms	Primers (5'–3')	Restriction enzyme
EGF	G61A (rs4444903)	Forward: TGCTACTAAAGGAAAGGA Reverse: TTCACAGAGTTTAAACAGCCC	<i>AluI</i>
TGFA	<i>TaqI</i> (rs11466267)	Forward: TTGTTTTGTTTTTGAGACGG Reverse: GTGTGAGACTTTCCAGCCCTGT	<i>TaqI</i>
	<i>RsaI</i> (rs3732248)	Forward: TGCCTCACACGACAGACACA Reverse: TGAATAACCCCAAGCAGACGG	<i>RsaI</i>
	<i>BamHI</i> (rs11466297)	Forward: ACAGATGGCGGAAGCAGAGGT Reverse: CTAAAGGGCAAGGAAACACAG	<i>BamHI</i>
	C3296T (rs2166975)	Forward: GCTCTGCCATCTCCAAGT Reverse: ATCTCTGGCAGTGCTGTCTT	—
	C3827T (rs1058213)	Forward: TGGGGAGAAAGTGAAGGAG Reverse: ATCTCCAAGGGTGGCGATAG	—
TS	28-bp repeat in enhancer region (rs45445694)	Forward: GTGGCTCCTGCGTTTCCCC Reverse: GCTCCGAGCCGGCCACAGGCATGGCGCGG	—
	G/C SNP in second repeat (rs2853542)	Forward: GTGGCTCCTGCGTTTCCCC Reverse: GCTCCGAGCCGGCCACAGGCATGGCGCGG	<i>HaeIII</i>
	6-bp deletion in 3'-UTR (rs16430)	Forward: CAAATCTGAGGGAGCTGAGT Reverse: CAGATAAGTGGCAGTACAGA	<i>DraI</i>
ERCC1	Asn118Asn (rs11615)	Forward: TCATCCCTATTGATGGCTTCTGCC Reverse: GACCATGCCAGAGGCTTCTCATAG	<i>BsrDI</i>
	C8092A (rs3212986)	Forward: CAGAGACAGTGGCCCAAGAG Reverse: GGGCACCTTCAGCTTTCTTT	<i>MboII</i>
XPD	Arg156Arg (rs238406)	Forward: CACACCTGGCTCATTTTTGTAT Reverse: TCATCCAGTTGTAGATGCCA	<i>TfiI</i>
	Asp312Asn (rs1799793)	Forward: CTGTTGGTGGGTGCCGTATCTGTTGGTCT Reverse: (TAATA)TCGGGGCTCACCTGCAGCACTTCC	<i>StyI</i>
FCGR2A	His166Arg (rs1801274)	Forward: GGAAAATCCCAGAAATTCTCCG Reverse: CAGCGTGTAGCCTATGTTTCC	<i>BstUI</i>
FCGR3A	Val212Phe (rs396991)	Forward: TGGCAAAGGCAGGAAGTATT Reverse: ATTGCAGTTCCACACACAG	—

EGF, epidermal growth factor; ERCC1, excision repair cross-complementation group 1; FCGR, fragment c gamma receptor; SNP, single nucleotide polymorphism; TGFA, transforming growth factor- α ; TS, thymidylate synthase; UTR, untranslated region; XPD, *Xeroderma pigmentosum* group D.

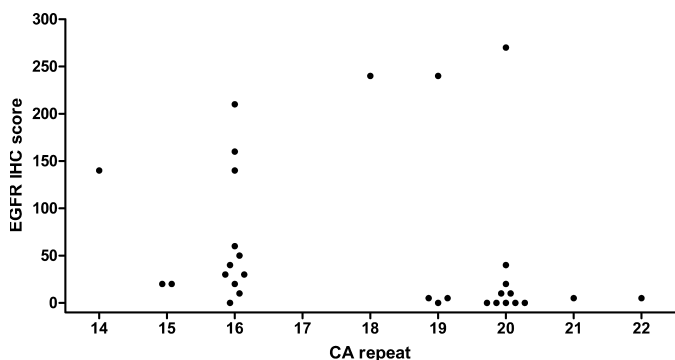


Fig. 1. CA repeat length and tissue epidermal growth factor receptor (EGFR) expression. Tissue EGFR expression determined by immunohistochemistry was analyzed according to CA repeat length in the remaining allele in patients with at least one 20 repeat allele. Immunohistochemistry (IHC) score was derived by multiplying the staining intensity and percentage of positive cells. Spearman's ρ -value was -0.46 ($p = 0.010$).

In order to examine whether the longer PFS and OS in patients with low CA repeat was due to an innate good prognosis associated with low CA repeat regardless of cetuximab treatment, we evaluated the impact of the CA repeat status in a separate AGC patient cohort treated only with modified FOLFOX6.⁽¹⁶⁾ None of these patients received EGFR-targeted treatment after disease progression. Sixty-eight patients were assessable for CA repeat statuses. There was no significant difference in the response rate (37% in low repeat and 46.3% in

high repeat, $P = 0.45$) and PFS (median 6.1 months in low repeat and 5 months in high repeat, $P = 0.33$). The OS was not different between the two groups (median 14 months in low repeat and 15.3 months in high repeat, $P = 0.74$; Fig. 2B). In the multivariate backward stepwise Cox regression analysis of PFS and OS for the adjustment of baseline characteristics, the CA repeat status was removed during the stepwise analysis. In summary, the CA repeat status was associated with survival only in patients who received cetuximab plus chemotherapy, but not in patients who were treated only with chemotherapy.

Ligand polymorphisms. The genotype frequency of EGF G61A single nucleotide polymorphisms (SNP) was GG in 20 patients, GA in 17, and AA in one. There was no association between the presence of the A allele and baseline characteristics. The serum EGF level was not different between the GG and GA/AA genotypes ($P = 0.75$ by Mann-Whitney U -test). Response, PFS, and OS were not affected by EGF G61A SNP.

Among the transforming growth factor- α (TGF- α) polymorphisms, all patients had the AA genotype in the *BamHI* SNP site. Genotypes in the *RsaI*, C3296T, and C3827T SNP sites were completely identical. The genotype was CC in 20 patients, CT in 13, and TT in five. No association was found between the genotype and baseline characteristics. There was no significant difference in the serum TGF- α level between the three genotypes ($P = 0.37$ by Kruskal-Wallis test). The response rate, PFS, and OS were not significantly different between the CC and CT/TT genotype. In the *TaqI* polymorphic site of TGF- α , 32 patients had the TAAT/TAAT (C1C1) genotype, and six patients had TAAT/- (C1C2) genotype. There was no significant association between the genotype and baseline

Table 3. Genetic polymorphisms and treatment outcomes

	Criteria (no. patients)	Responders (%)	<i>P</i> -value*	Median PFS (months)	<i>P</i> -value**	Median OS (months)	<i>P</i> -value**
EGFR CA-SSR†	≤37 (21)	10 (58.8)	0.69	5.5	0.64	14.4	0.22
	≥38 (17)	11 (52.4)		5.3		7.6	
EGF 61	GG (20)	11 (55.0)	0.97	5.5	0.77	13.5	0.38
	GA, AA (18)	10 (55.6)		5.5		8.2	
TGF-α RsaI	CC (20)	10 (50.0)	0.49	5.6	0.55	16.9	0.60
	CT, TT (18)	11 (61.1)		5.5		8.5	
TGF-α TaqI	TAAT/TAAT(32)	18 (56.3)	1.00	5.5	0.87	9.9	0.81
	TAAT/- (6)	3 (50.0)		5.3		8.2	
FCGR2A	HH (21)	14 (66.7)	0.12	5.6	0.61	13.5	0.89
	HR, RR (17)	7 (41.2)		5.5		9.9	
FCGR3A	VV (18)	9 (50)	0.54	5.3	0.22	9.9	0.89
	VF (20)	12 (60)		5.5		9.2	
TSER	3R/3R (28)	16 (57.1)	0.73	5.5	0.91	16.9	0.12
	2R/2R, 2R/3R (10)	5 (50)		5.5		5.6	
TSER‡	High type (27)	13 (48.1)	0.28	5.5	0.50	18.8	0.024
	Low type (11)	8 (72.7)		5.5		8.5	
TS 3'-UTR	-6 bp/-6 bp (21)	11 (52.4)	0.69	5.5	0.41	16.9	0.65
	+6 bp/+6 bp, +6 bp/-6 bp (17)	10 (59.8)		5.5		9.9	
ERCC1 118	CC (23)	12 (52.2)	0.64	5.6	0.81	14.4	0.94
	CT, TT (15)	9 (60)		5.0		9.2	
ERCC1 8092	CC (28)	15 (53.6)	1.00	5.5	0.64	9.9	0.13
	AA, AC (10)	6 (60)		5.3		8.5	
XPD 156	CC (16)	9 (56.3)	0.92	5.6	0.53	7.6	0.35
	AA, AC (22)	12 (54.5)		5.5		9.9	

P*-values by chi-squared test or Fisher's exact test; *P*-values by log-rank test; †sum of number of repeats in both alleles; ‡high type includes 2R/3G, 3C/3G, and 3G/3G; low type includes 2R/2R, 2R/3C, and 3C/3C. CA-SSR, CA simple sequence repeat; EGF, epidermal growth factor; ERCC1, excision repair cross-complementation group 1; FCGR, fragment c gamma receptor; TGF-α, transforming growth factor-α; TS, thymidylate synthase; TSER, thymidylate synthase enhancer region; UTR, untranslated region; XPD, *Xeroderma pigmentosum* group D.

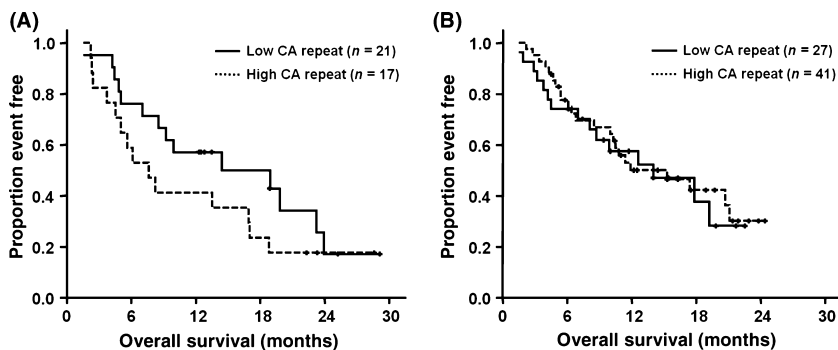


Fig. 2. Kaplan-Meier curves of overall survival in patients treated with cetuximab plus modified leucovorin, fluorouracil, and oxaliplatin 6 (mFOLFOX6) (A) and in another cohort of patients treated only with mFOLFOX6 (B). Adjusted hazard ratio (adjusted HR) and *P*-values were calculated with the backward stepwise Cox regression analysis with baseline characteristics as covariates. (A) Adjusted HR 0.40 (0.16–0.99), *P* = 0.048. (B) *P* = not significant. Adjusted HR and *P*-value are not given in patients treated only with mFOLFOX6 because the CA repeat status was removed during the stepwise analysis.

characteristics or treatment outcomes. The serum TGF-α level was not significantly different in the two genotypes (*P* = 0.14 by Mann-Whitney *U*-test).

FCGR polymorphisms. The distribution of the FCGR2A polymorphism in H166R was HH in 21 patients, HR in 15, and RR in two patients. There was no difference in the baseline characteristics according to the genotype. There was a trend towards a higher response in the HH genotype patients compared with patients with the HR or RR genotype (adjusted odds ratio 3.40 [95% CI 0.82–14.08], *P* = 0.092). However, the difference in the response rate did not translate into difference in PFS or OS. There was no significant association between FCGR3A V212F SNP and baseline characteristics or treatment outcomes.

Polymorphisms related to 5-FU and oxaliplatin. In the analysis of genotype and baseline characteristics, the XPD 156 A allele was associated with diffuse type cancer: 100% (6/6) in the AA genotype, 75% (12/16) in the AC genotype, and 50% (8/16) in

the CC genotype (*P* = 0.020 by linear-by-linear association). Patients with the 2R allele in the thymidylate synthase enhancer region (TSER) or low-type TSER genotype more frequently had liver metastasis (70% in 2R/2R or 2R/3R vs 28.6% in 3R/3R, *P* = 0.030; 72.7% in low type [2R/2R, 2R/3C, and 3C/3C] vs 25.9% in high type (2R/3G, 3C/3G, and 3G/3G), *P* = 0.012). In contrast, patients with the -6-bp/-6-bp genotype in the TS 3'-UTR had a lower frequency of liver metastasis (23.8% in -6 bp/-6 bp vs 58.8% in +6 bp/+6 bp or +6 bp/-6 bp, *P* = 0.028).

Patients with the high-type TSER genotype had a significantly longer OS compared to those with a low-type genotype (*P* = 0.024 by log-rank test; Table 3), but this was not statistically significant in the multivariate analysis adjusting for the baseline characteristics listed earlier. The TSER genotype was removed during the stepwise analysis, whereas older age (HR 0.47, 95% CI 0.21–1.04), peritoneal seeding (HR 3.04, 95% CI

Table 4. Multivariate analysis of survival

	Criteria (no. patients)	Adjusted hazard ratio (95% confidence interval)	P-value
Progression-free survival			
EGFR CA-SSR	≤37 (21)	0.42 (0.19–0.96)	0.040
	≥38 (17)	1	
Sex	Male (28)	1	0.050
	Female (10)	2.39 (1.00–5.69)	
Age	<56.5 years (19)	1	<0.001
	>56.5 years (19)	0.17 (0.066–0.45)	
Performance status (ECOG)	0 (7)	1	0.001
	1–2 (31)	7.30 (2.27–23.5)	
Overall survival			
EGFR CA-SSR	≤37 (21)	0.40 (0.16–0.99)	0.048
	≥38 (17)	1	
Age	<56.5 years (19)	1	0.020
	>56.5 years (19)	0.37 (0.16–0.86)	
Peritoneal seeding	No (19)	1	0.002
	Yes (19)	4.15 (1.68–10.2)	
Liver metastasis	No (23)	1	0.003
	Yes (15)	3.57 (1.55–8.24)	

Multivariate analysis was performed using the backward stepwise Cox regression model. Covariates entered were epidermal growth factor receptor (EGFR) CA simple sequence repeat (CA-SSR) (sum ≤37 vs ≥38), sex, age (older vs younger than median), ECOG PS (0 vs 1–2), Lauren classification, peritoneal seeding in the progression-free survival analysis and EGFR CA-SSR, sex, age, Eastern Cooperative Oncology Group (ECOG) performance status, Lauren classification, peritoneal seeding, liver metastasis, and number of organs involved (1–2 vs ≥3) in the overall survival analysis. Covariates in the final models are shown.

1.35–6.87), and liver metastasis (HR 4.09, 95% CI 1.79–9.36) were in the final model. In the case of ERCC1 C8092A SNP, patients with the CC genotype had a longer OS compared with those with the CA or AA genotype (adjusted HR 0.39 [95% CI 0.15–0.97], $P = 0.044$). However, ERCC1 C8092A was not retained in the stepwise multivariate analysis with baseline characteristics and CA repeat status as covariates, whereas CA repeat statuses remained in the final model.

Discussion

The identification of a predictive biomarker has become an important issue in the era of molecular-targeted treatment. Treatment that is effective in a certain subgroup of patients can be futile in others. In the case of cetuximab in colorectal cancer, adding cetuximab to first-line chemotherapy can only improve the treatment outcome in patients with K-ras wild-type tumors.^(7,18) In contrast, there was no additional benefit of cetuximab compared with chemotherapy alone in patients with K-ras mutant tumors.^(7,18) Therefore, cetuximab is recommended for colorectal cancer patients only if they have a K-ras wild-type tumor.⁽⁹⁾ Other biomarkers potentially associated with the efficacy of cetuximab in colorectal cancer include B-raf mutation, phosphatidylinositol 3-kinase/phosphatase and tensin homolog (PI3KCA/PTEN) deregulation, EGFR gene amplification, EGFR ligand (epiregulin and amphiregulin) expression, and polymorphisms in EGFR, EGF, and FCGR.^(19–25)

Trastuzumab, an anti-HER2 antibody, has recently shown activity in AGC.⁽⁴⁾ Trastuzumab was the first molecular-targeted agent to prove efficacy in gastric cancer, opening a new era of gastric cancer treatment. Patient inclusion of the study was limited to HER2-positive tumors, which could have been the key to the study's success. Whether cetuximab can improve the treat-

ment outcome of gastric cancer will be addressed by the ongoing phase III study.⁽¹²⁾ However, patients are not selected based on molecular markers in the cetuximab study. In fact, there is no biomarker to predict the differential effect of cetuximab in gastric cancer. K-ras mutation, which is a good predictor of the lack of benefit from cetuximab in colorectal cancer, is an uncommon genetic event in gastric cancer.^(13,14,26)

In order to find candidates for a predictive biomarker of cetuximab efficacy in gastric cancer, we performed an exploratory biomarker analysis in a phase II study of cetuximab. In our previous study focusing on tumor tissue and serum, patients with an EGFR expression and low ligand levels had better outcomes with cetuximab/mFOLFOX6 treatment.⁽¹⁴⁾ In the present study, germline genetic polymorphisms analyzed with peripheral blood mononuclear cell DNA are presented.

A shorter repeat of the EGFR intron 1 CA dinucleotide SSR polymorphism has been associated with poor survival in non-small cell lung cancer and pancreatic cancer, suggesting its association with poor prognosis.^(27,28) In contrast, a short CA repeat was associated with better treatment results with either the EGFR tyrosine kinase inhibitor (gefitinib and erlotinib) or anti-EGFR monoclonal antibody (cetuximab).^(17,25,29,30) We have previously reported that a low CA repeat was associated with a better response in non-small cell lung cancer patients treated with gefitinib, independent of EGFR mutational status.⁽¹⁷⁾ Because there is no general agreement in the cut-off of CA repeats, we applied the same cut-off (sum ≤37 vs ≥38) that was used in our previous study of gefitinib in non-small cell lung cancer and found that a shorter CA repeat was independently associated with longer survival in AGC patients treated with cetuximab. Collectively, it is possible that the EGFR intron 1 CA repeat polymorphism is a common predictive biomarker for the treatment outcome of EGFR-targeted agents in various types of cancers. In contrast, the CA repeat status did not impact survival among AGC patients not receiving cetuximab. This finding suggests that this polymorphism could be a predictive biomarker of cetuximab efficacy in gastric cancer, which merits further investigation in randomized studies. Moreover, as this study is the first to examine the polymorphism in gastric cancer, further studies regarding its prognostic role in gastric cancer is warranted.

The CA repeat polymorphism of EGFR has interethnic variability that Asians have higher repeat numbers compared with Caucasians.^(27,31,32) In the present study, a 20 repeat allele was the most frequent allele, and the distribution of repeat length was similar to that of Asian patients in previous studies.^(27,31,32) The longer repeat length in Korean patients could be one of the reasons why cetuximab/mFOLFOX6 showed disappointing results in previous study.⁽¹⁴⁾ In contrast, phase II studies of cetuximab in gastric cancer performed in Caucasian patients, who generally have shorter CA repeats, showed positive results.^(10,11) Therefore, it would be interesting to determine whether the interethnic difference in CA repeat distribution could affect the treatment outcome of cetuximab in different ethnicities. Intriguingly, the benefit of cetuximab was evident among Caucasians, whereas Asian patients did not benefit from cetuximab in a phase III study of cetuximab plus chemotherapy in non-small cell lung cancer.⁽³³⁾ Although other factors, such as imbalance in subsequent treatment, might have led to such a difference, interethnic genetic difference could also be considered.

The inverse association between the CA repeat number and tumor EGFR expression is in line with previous reports in breast and head and neck cancers, showing a higher EGFR expression in low CA repeats.^(34,35) We could not find association between genetic polymorphisms and tumor expression or serum level in other polymorphisms.

The biomarker selection for mFOLFOX6 was based on our previous pharmacogenomic study.⁽¹⁶⁾ However, the

polymorphisms associated with poor response to mFOLFOX6 (TS 3'-UTR and XPD 156) were not associated with outcome in this study.⁽¹⁶⁾ It is possible that the addition of cetuximab to mFOLFOX6 increased the response rates in patients with poor response alleles. This possibility could only be examined in a randomized study. The ERCC1 C8092A SNP was the only mFOLFOX6-related polymorphism associated with outcome in the adjusted analysis in the present study. It has been reported that patients with the CC genotype have a longer OS compared to those with the CA or AA genotype in other cancers treated with platinum-containing chemotherapy.^(36,37) However, the association was not significant in the multivariate analysis, with the EGFR CA repeat status as a covariate.

The present study was an exploratory biomarker study performed in a small-sized single-arm phase II study. Therefore, whether the EGFR intron 1 CA repeat polymorphism can predict

the benefits of cetuximab in gastric cancer needs to be investigated in a randomized study.

In conclusion, a low repeat of the EGFR intron 1 CA repeat polymorphism was associated with longer survival in AGC patients treated with cetuximab plus mFOLFOX6, but not in patients treated only with mFOLFOX6.

Acknowledgments

This study was supported in part by grants from the Korean Healthcare Technology R&D project (No. A091081) and the Korea Health 21 R&D project (No. A030001), Ministry for Health, Welfare and Family Affairs, Korea. We thank Ms Hye Seon Ham and Ms Soo Jin Park (Cancer Research Institute, Seoul National University, Seoul, Korea) for their technical expertise in the genotype analysis.

References

- Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol* 2006; **24**: 2137–50.
- Shin HR, Jung KW, Won YJ *et al*. National cancer incidence for the year 2002 in Korea. *Cancer Res Treat* 2007; **39**: 139–49.
- Ohtsu A. Chemotherapy for metastatic gastric cancer: past, present, and future. *J Gastroenterol* 2008; **43**: 256–64.
- Van Cutsem E, Kang Y, Chung H *et al*. Efficacy results from the ToGA trial: a phase III study of trastuzumab added to standard chemotherapy (CT) in first-line human epidermal growth factor receptor 2 (HER2)-positive advanced gastric cancer (GC). *J Clin Oncol (Meeting Abstracts)* 2009; **27**: LBA4509.
- Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol* 2003; **21**: 2787–99.
- Jonker DJ, O'Callaghan CJ, Karapetis CS *et al*. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007; **357**: 2040–8.
- Van Cutsem E, Kohne C-H, Hitre E *et al*. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009; **360**: 1408–17.
- Karapetis CS, Khambata-Ford S, Jonker DJ *et al*. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008; **359**: 1757–65.
- Allegra CJ, Jessup JM, Somerfield MR *et al*. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009; **27**: 2091–6.
- Pinto C, Di Fabio F, Siena S *et al*. Phase II study of cetuximab in combination with FOLFIRI in patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma (FOLCETUX study). *Ann Oncol* 2007; **18**: 510–7.
- Lordick F, Lorenzen S, Hegewisch-Becker S *et al*. Cetuximab plus weekly oxaliplatin/5FU/FA (FUFOX) in 1st line metastatic gastric cancer. Final results from a multicenter phase II study of the AIO upper GI study group. *J Clin Oncol (Meeting Abstracts)*. 2007; **25**: abstract 4526.
- Erbixituz in combination with Xeloda and cisplatin in advanced esophago-gastric cancer (EXPAND) (ClinicalTrials.gov number, NCT00678535).
- Lee SH, Lee JW, Soung YH *et al*. BRAF and KRAS mutations in stomach cancer. *Oncogene* 2003; **22**: 6942–5.
- Han SW, Oh DY, Im SA *et al*. Phase II study and biomarker analysis of cetuximab combined with modified FOLFOX6 in advanced gastric cancer. *Br J Cancer* 2009; **100**: 298–304.
- Therasse P, Arbuck SG, Eisenhauer EA *et al*. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205–16.
- Keam B, Im SA, Han SW *et al*. Modified FOLFOX-6 chemotherapy in advanced gastric cancer: results of phase II study and comprehensive analysis of polymorphisms as a predictive and prognostic marker. *BMC Cancer* 2008; **8**: 148.
- Han SW, Jeon YK, Lee KH *et al*. Intron 1 CA dinucleotide repeat polymorphism and mutations of epidermal growth factor receptor and gefitinib responsiveness in non-small-cell lung cancer. *Pharmacogenet Genomics* 2007; **17**: 313–9.
- Bokemeyer C, Bondarenko I, Makhson A *et al*. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 2009; **27**: 663–71.
- Di Nicolantonio F, Martini M, Molinari F *et al*. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 5705–12.
- Perrone F, Lampis A, Orsenigo M *et al*. PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. *Ann Oncol* 2009; **20**: 84–90.
- Moroni M, Veronese S, Benvenuti S *et al*. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 2005; **6**: 279–86.
- Cappuzzo F, Finocchiaro G, Rossi E *et al*. EGFR FISH assay predicts for response to cetuximab in chemotherapy refractory colorectal cancer patients. *Ann Oncol* 2008; **19**: 717–23.
- Khambata-Ford S, Garrett CR, Meropol NJ *et al*. Expression of epi-regulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007; **25**: 3230–7.
- Zhang W, Gordon M, Schultheis AM *et al*. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. *J Clin Oncol* 2007; **25**: 3712–8.
- Graziano F, Ruzzo A, Loupakis F *et al*. Pharmacogenetic profiling for cetuximab plus irinotecan therapy in patients with refractory advanced colorectal cancer. *J Clin Oncol* 2008; **26**: 1427–34.
- Stella G, Rojas Llimpe F, Barone C *et al*. KRAS and BRAF mutational status as response biomarkers to cetuximab combination therapy in advanced gastric cancer patients. *J Clin Oncol (Meeting Abstracts)* 2009; **27**: e15503.
- Nomura M, Shigematsu H, Li L *et al*. Polymorphisms, mutations, and amplification of the EGFR gene in non-small cell lung cancers. *PLoS Med* 2007; **4**: e125.
- Tzeng CW, Frolov A, Frolova N *et al*. Pancreatic cancer epidermal growth factor receptor (EGFR) intron 1 polymorphism influences postoperative patient survival and in vitro erlotinib response. *Ann Surg Oncol* 2007; **14**: 2150–8.
- Amador ML, Oppenheimer D, Perea S *et al*. An epidermal growth factor receptor intron 1 polymorphism mediates response to epidermal growth factor receptor inhibitors. *Cancer Res* 2004; **64**: 9139–43.
- Liu G, Gurubhagavatula S, Zhou W *et al*. Epidermal growth factor receptor polymorphisms and clinical outcomes in non-small-cell lung cancer patients treated with gefitinib. *Pharmacogenomics J* 2008; **8**: 129–38.
- Liu W, Innocenti F, Chen P, Das S, Cook EH Jr, Ratain MJ. Interethnic difference in the allelic distribution of human epidermal growth factor receptor intron 1 polymorphism. *Clin Cancer Res* 2003; **9**: 1009–12.
- Buerger H, Packeisen J, Boecker A *et al*. Allelic length of a CA dinucleotide repeat in the egfr gene correlates with the frequency of amplifications of this sequence – first results of an inter-ethnic breast cancer study. *J Pathol* 2004; **203**: 545–50.
- Pirker R, Pereira JR, Szczesna A *et al*. Cetuximab plus chemotherapy in patients with advanced non-small-cell lung cancer (FLEX): an open-label randomised phase III trial. *Lancet* 2009; **373**: 1525–31.
- Buerger H, Gebhardt F, Schmidt H *et al*. Length and loss of heterozygosity of an intron 1 polymorphic sequence of egfr is related to cytogenetic alterations and epithelial growth factor receptor expression. *Cancer Res* 2000; **60**: 854–7.
- Etienne-Grimaldi MC, Pereira S, Magne N *et al*. Analysis of the dinucleotide repeat polymorphism in the epidermal growth factor receptor (EGFR) gene in head and neck cancer patients. *Ann Oncol* 2005; **16**: 934–41.
- Zhou W, Gurubhagavatula S, Liu G *et al*. Excision repair cross-complementation group 1 polymorphism predicts overall survival in advanced

non-small cell lung cancer patients treated with platinum-based chemotherapy. *Clin Cancer Res* 2004; **10**: 4939–43.

37 Krivak TC, Darcy KM, Tian C *et al.* Relationship between ERCC1 polymorphisms, disease progression, and survival in the Gynecologic

Oncology Group Phase III Trial of intraperitoneal versus intravenous cisplatin and paclitaxel for stage III epithelial ovarian cancer. *J Clin Oncol* 2008; **26**: 3598–606.

Supporting Information

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

Table S1. Baseline characteristics of modified leucovorin, fluorouracil, and oxaliplatin 6 (mFOLFOX6)-only cohort.

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