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EGF61 Polymorphism Predicts Complete Pathologic Response to Cetuximab-Based Chemoradiation Independent of KRAS Status in Locally Advanced Rectal Cancer Patients

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Abstract

Background: Cetuximab has shown significant clinical activity in metastatic colon cancer. However, cetuximab-containing neoadjuvant chemoradiation has not been shown to improve tumor response in locally advanced rectal cancer patients in recent phase I/II trials. We evaluated functional germline polymorphisms of genes involved in epidermal growth factor receptor pathway, angiogenesis, antibody-dependent cell-mediated cytotoxicity, DNA repair, and drug metabolism, for their potential role as molecular predictors for clinical outcome in locally advanced rectal cancer patients treated with preoperative cetuximab-based chemoradiation.

Methods: 130 patients (74 men and 56 women) with locally advanced rectal cancer (4 with stage II, 109 with stage III, and 15 with stage IV, 2 unknown) who were enrolled in phase I/II clinical trials treated with cetuximab-based chemoradiation in European cancer centers were included. Genomic DNA was extracted from formalin-fixed paraffin-embedded tumor samples and genotyping was done by using PCR-RFLP assays. Fisher's exact test was used to examine associations between polymorphisms and complete pathologic response (pCR) that was determined by a modified Dworak classification system (grade III vs. grade IV: complete response).

Results: Patients with the epidermal growth factor (EGF) 61 G/G genotype had pCR of 45% (5/11), compared with 21% (11/53) in patients heterozygous, and 2% (1/54) in patients homozygous for the A/A allele ($P < 0.001$). In addition, this association between EGF 61 G allele and pCR remained significant ($P = 0.019$) in the 59 patients with wild-type KRAS.

Conclusion: This study suggested EGF A+61G polymorphism to be a predictive marker for pCR, independent of KRAS mutation status, to cetuximab-based neoadjuvant chemoradiation of patients with locally advanced rectal cancer. *Clin Cancer Res*; 17(15); 5161–9. ©2011 AACR.

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Introduction

It is estimated that 142,570 Americans will be diagnosed with colorectal cancer (CRC) in 2010, of which 39,670 are rectal cancers. The incidence rate of rectal cancer has decreased from 18.42 to 12.77 per 100,000 over the past 20 years, largely attributed to the improved screening programs. From 1999 to 2006, 77% of diagnosed rectal cancer patients had localized or regional advanced disease and these patients had significant 5-year survival advantage (88.6% and 67.1%) compared with 12.3% for distantly metastasized patients (SEER's data). Similar trend has been observed in Western Europe. The widely used surgical technique, total mesorectal excision alone, has decreased the local recurrence rate from 39.4% to 9.8% and improved the observed 5-year survival rate from 50% to 71% (1). In addition, preoperative therapy with concurrent radiation

Translational Relevance

Our study suggests epidermal growth factor A+61G polymorphism is significantly associated with pathologic response to cetuximab-based neoadjuvant chemoradiation in locally advanced rectal cancer patients, independent of KRAS mutation status. This is the first study suggesting this polymorphism might be predictive marker for response to cetuximab-based therapy.

and infusion of 5FU has shown to further decrease the local recurrence rate in stages II and III rectal cancer (2–4). Therefore neoadjuvant chemoradiation is now a standard of care for locally advanced rectal cancer patients. Interestingly, neither pelvic radiation nor chemotherapy before surgery has shown significant survival benefits. Ongoing clinical trials are examining newer agents such as capecitabine, oxaliplatin, irinotecan, cetuximab, or bevacizumab, to further optimize local control, reduce distant metastases and ultimately improve survival.

Epidermal growth factor receptor (EGFR) signals through 2 major pathways, the RAS-mitogen-activated protein kinase and the PI3K-AKT pathway. Hyperactivity of the EGFR pathways is associated with more aggressive phenotype and poor prognosis of cancers (5) and can render cancer cells resistant to radiation (6). Cetuximab is an EGFR monoclonal antibody and has shown significant clinical activity in metastatic colon cancer (7, 8). In addition, concomitant high-dose radiotherapy plus cetuximab has been shown to improve locoregional control and reduce mortality in squamous carcinoma of the head and neck (9). Recent phase I/II clinical trials have evaluated EGFR inhibitors as a radiosensitizer in CRC and showed that cetuximab can be safely given without unexpected toxicity (10, 11). However, cetuximab-containing neoadjuvant chemoradiation has not been shown to improve tumor response and complete pathologic response (pCR) rates in locally advanced rectal cancer patients (12–14). To identify the subgroup of patients who may benefit from the cetuximab protocol, we designed a retrospective study within a cohort of 4 prospectively conducted phase I/II clinical trials. We evaluated functional germline polymorphisms of genes involved in the EGFR signaling pathway (EGF, EGFR, COX-2, KRAS, CyclinD1), angiogenesis (VEGF, IL-8), antibody-dependent cell-mediated cytotoxicity (FCGR2A/3A), DNA repair (XRCC3, Rad51), and drug metabolism (TS, MTHFR), for their potential role as molecular predictors for clinical outcome in locally advanced rectal cancer patients treated with preoperative cetuximab-based chemoradiation followed by surgery.

Materials and Methods

Patients

130 patients (74 men and 56 women, median age 61) with locally advanced rectal cancer (4 with stage II, 109 with stage III, and 15 with stage IV, 2 unknown) who were

enrolled in phase I/II clinical trials treated with cetuximab-based chemoradiation in 4 European cancer centers (Brussels/Leuven, Belgium; Ljubljana, Slovenia; Halle/Erlangen/Göttingen/Regensburg, Germany, and Cologne, Germany) were included (Table 1; refs. 15, 16). Informed consent was obtained from each patient and the studies were approved by the Institutional Review Boards of the participating centers. Clinical staging was based on results from endoscopy, endoscopic ultrasound, and computed tomography/MRI of the chest and abdomen. Patients underwent radical resection within 4 to 8 weeks after completion of chemoradiation by either a low anterior resection or an abdomino-perineal rectal amputation with total mesorectal excision.

Treatment protocols

Brussels/Leuven, Belgium. Patients ($n = 41$) received capecitabine (4 patients at first dose level of 650 mg/m² twice a day; 37 patients at second dose level of 825 mg/m² twice a day) and intravenous cetuximab (400 mg/m² as initial dose 1 week before CRT followed by 250 mg/m²/week for 5 weeks) and 45 Gy of radiation (25×1.8 Gy).

Ljubljana, Slovenia. Patients ($n = 31$) received capecitabine 1,250 mg/m² twice daily for 2 weeks followed by intravenous cetuximab 400 mg/m² at week 3, then weekly intravenous 250 mg/m² cetuximab plus CRT including capecitabine 825 mg/m² twice daily (including weekends during radiotherapy) with radiotherapy of 45 Gy (25×1.8 Gy), 5 days a week for 5 weeks.

Halle/Erlangen/Göttingen/Regensburg, Germany. Patients ($n = 42$) received cetuximab given as an initial dose of 400 mg/m² 7 days before the start of RT, and then at 250 mg/m² once weekly during RT (50.4 Gy, 28×1.8 Gy).

Table 1. Patient characteristics and treatments

	N = 130	%
Age, y		
Median	61	
Range	33–83	
Sex		
Male	74	57
Female	56	43
Stage		
II	4	3
III	109	85
IV	15	12
Type of neoadjuvant therapy		
5-FU/Cetuximab/Radiation (Cologne, Germany)	16	12
Capecitabine/Cetuximab/ Radiation (Belgium/Slovenia)	72	56
Capecitabine/Oxaliplatin/ Cetuximab/Radiation (Halle/Erlangen/Göttingen/Regensburg, Germany)	42	32

Capecitabine and oxaliplatin were administered according to an established schedule of oxaliplatin (50 mg/m² on Days 1, 8, 22, and 29) and capecitabine (Days 1–14 and 22–35) at 3 dose levels: 1,000, 1,300, and 1,650 mg/m²/d.

Cologne, Germany. Patients ($n = 16$) were treated with 45 Gy of radiation (25 × 1.8 Gy) and cetuximab (400 mg/m² as initial dose 1 week before CRT followed by 250 mg/m²/wk for 5 weeks) in combination with 5-FU.

Tissue samples and pCR evaluation

Tumor biopsies from the study patients were obtained at the time of diagnosis (pretherapeutic biopsy), and at the time of surgery (posttherapeutic biopsy). Pretreatment biopsies from 109 (84%) patients and posttreatment biopsies from 117 (90%) patients were collected and analyzed in this study. For diagnosis, 3 sections representative of the beginning, middle, and end of the tissue were collected. The resected specimens were fixed in 10% formalin, embedded in paraffin, and cut into 5-μm thick slices. The sections were then stained with hematoxylin and eosin stain and all samples were reviewed by a staff pathologist in each study center. To determine pCR, Dworak's regression grading system (grade 0: no regression; grade 1: minimal regression; grade 2: moderate regression; grade 3: good regression; and grade 4: total regression) was used in all of the participating centers (17). Grade 4 was defined as complete pCR.

DNA extraction

Formalin-fixed paraffin-embedded tissues from the patients were dissected as described previously. Ten micrometer thick slides were obtained from the identified areas with the highest tumor concentration and were mounted

on uncoated glass slides. Sections were deparaffinized in xylene for 10 minutes, hydrated with 100%, 95%, and 70% ethanol and then washed in H₂O for 30 seconds. The sections were then stained with nuclear fast red (American Master Tech Scientific, Inc.) for 20 seconds and rinsed in H₂O for 30 seconds, dehydrated with 70% ethanol, 95% ethanol, and 100% ethanol for 30 seconds each, followed by xylene for 10 minutes and then completely air-dried. Laser capture microdissection (P.A.L.M. Microlaser Technologies AG) was used in all tumor samples. Genomic DNA was then extracted from the dissected tumor samples by using the QIAamp kit (Qiagen) according to manufacturer's instructions.

KRAS analysis

KRAS analysis of 7 KRAS mutations (codon 12 and codon 13) was done according to a proprietary procedure defined by Response Genetics Inc. (United States patent number 6, 248, 535).

PCR-RFLP

Genotyping was done by using PCR-RFLP assays. Briefly, forward primers and reverse primers (Table 2) were used for PCR amplification, PCR products were digested by restriction enzymes (New England Biolab), and alleles were separated on 4% NuSieve ethidium bromide-stained agarose gel.

Statistical analysis

Tumor response to neoadjuvant chemoradiotherapy evaluated by Dworak criteria was the primary endpoint. Patients with grade 4 Dworak response were classified as

Table 2. Primers and enzymes for PCR-RFLP

Gene	Forward-Primer (5'-3')	Reverse-Primer (5'-3')	Enzyme	Annealing
FCGR2A H131R	GGAAATCCCAGAAATTCCTCGC	CAACAGCCTGACTACCTATTACGCGGG	BstU I	55°
FCGR3A V158F	CTGAAGACACATTTTACTCCAAA/C	TCCAAAAGCCACACTCAAAGAC	n.a.	64°
VEGF C+936T	AAGGAAGAGGAGACTCTGCGCAGAG C	TAAATGTATGTATGTGGGTGGGTGTGT CTACAG G	Nla III	60°
EGF A+61G	CATTTGCAAACAGAGGCTCA	TGTGACAGAGCAAGGCAAAG	Alu I	60°
EGFR (CA) _n Repeat	ACCCAGGGCTCTATGGGAA	TGAGGGCACAAGAAGCCCCT	n.a.	55°
EGFR G+497A	TGCTGTGACCCACTCTGTCT	CCAGAAGGTTGCACTTGTC	Bst-N I	59°
MTHFR C677T	GCACTTGAAGGAGAAGGTGTCT	TGTGTCAGCCTCGAAAGAAAAGCT	Hin fl	68°
IL8 T-251A	TTGTTCTAACACCTGCCACTCT	GGCAAACCTGAGTCTCACA	Mfe I	60°
MTHFR A-1298C	GGAGGAGCTGCTGAAGATGTG	CCCGAGAGGTAAAGAACAAGACTT	Mbo II	68°
COX-2 G-765C	ATTCTGGCCATCGCCGCTTC	CTCCTTGTTCCTTGGAAG GAGACG	Aci I	55°
Cyclin D1A+870G	GTGAAGTTCATTCCAATCCGC	GGGACATCACCCTCACTTAC	ScrF I	61°
COX-2 G-765C	ATTCTGGCCATCGCCGCTTC	CTCCTTGTTCCTTGGAAG GAGACG	Aci I	55°
RCC3Thr241 Met	GCCTGGTGGTCATCGACTC	ACAGGGCTCTGGAAGGCACTGCTCA GCTCACGC ACC	Nco I	60°
S 3'UTR 6bp+/-	CAAATCTGAGGGAGCTGAGT	CAGATAAGTGGCAGTACAGA	Dra I	58°
S 5'UTR 2R/3R	GAAAAGGCGCGCGGAAGG	GCTCCGAGCCGCGCCACA	n.a	62°
RAD G51C	TGGGAAGTCAACTCATCTGG	GCGCTCCTCTCTCCAGCAG	Mva I	60°
KRAS let-7 lcs6	TGACTGAATATAAACTTGTGGTAGTT G	TCGTCCACAAAATGATTCTGAA	Tfi I	60°

having pCR. Patients having grade 0 to 3 Dworak response were categorized as not having pCR. Fisher's exact test was used to examine associations between polymorphisms and pCR or KRAS mutation status and pCR. The exact conditional test was used to evaluate the associations between polymorphisms and ordinal Dworak response. The independent effects of polymorphisms on pCR were examined by using the Logistic regression model when stratifying the study center and patient baseline characteristics.

Although the genetic model of inheritance for the polymorphisms had not established, a codominant model was considered first. A dominant model was used whenever the patients carrying the genotype of variant/variant were too few (<5%).

The false discovery rate (FDR) of multiple testing was controlled by using the Benjamini and Hochberg method (18). The FDR adjusted *P* values less than 15% were considered as significant.

An internal validation method, leave-one-out cross validation was used to evaluate the process of polymorphism selection to predict pCR.

All statistical tests were 2-sided and done by using the SAS statistical package version 9.2 (SAS Institute Inc.).

Results

pCR

Data from 125 of 130 patients were available to evaluate pCR with cetuximab-based chemoradiation after surgery. Among them, 21 (17%) patients had grade 0 to 1, 55 (44%) patients had grade 2, 30 (24%) patients had grade 3, and 19 (15%) patients had grade 4 response.

EGF 61 polymorphism and complete pCR

Genotyping for EGF 61 was successful in 118 (91%) of 130 cases. In 12 patients (9%), genotyping was not successful because of limited quantity and quality of extracted genomic DNA or biopsy samples. Forty-six percent (54/118) of patients were homozygous for A/A allele, 45% (53/118) were heterozygous A/G, and 9% (11/118) were homozygous for the G/G allele. Patients with the EGF G/G genotype had a pCR of 45% (5/11), compared with 21% (11/53) in patients heterozygous, and 2% (1/54) in patients homozygous for the A/A allele, respectively (Fig. 1). There was statistically significant association between EGF A61G polymorphism and pCR after treatment of these patients ($P < 0.001$, Fisher's exact test, Table 3). EGF A61G polymorphism remained significantly associated with pCR in the multivariable logistic regression model stratified by the study center and age (Table 4).

TS 5'UTR polymorphism and complete pCR

Genotyping for TS 5'UTR was successful in 95 (73%) of 130 cases. In 35 patients (27%), genotyping was not successful because of limited quantity and quality of extracted genomic DNA or biopsy samples. Sixteen percent (15/95) of patients were homozygous for 2R/2R allele, 59% (56/95) were heterozygous 2R/3R, and 25% (24/

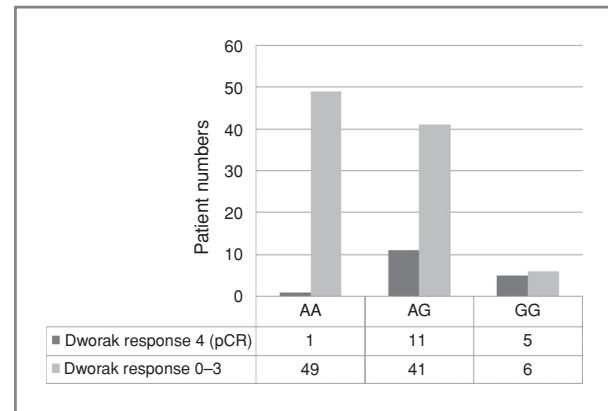


Figure 1. EGF 61 polymorphism status and pCR.

95) were homozygous for the 3R/3R allele. Patients with the TS 5'UTR 2R/2R genotype had a pCR of 33% (5/15), compared with 16% (9/56) in patients heterozygous, and 0% (0/24) in patients homozygous for the 3R/3R genotype, respectively. There was statistically significant association between TS 5'UTR 2R allele and pCR after treatment of these patients ($P = 0.006$, Fisher's exact test, Table 3). The association was no longer statistically significant in the multivariate logistic regression model (Table 4).

KRAS mutation status and pCR

KRAS mutation status was available from pretherapeutic tumor biopsies of 101 patients, 42% of which (42/101) had KRAS mutation, 58% (59/101) had wild-type KRAS. The mutation status (wild type vs. mutant) was not significantly correlated with pCR ($P = 0.214$, Fischer exact test). KRAS mutant patients showed 15% (5/34) pCR to cetuximab-based neoadjuvant CRT compared with KRAS wild-type patients with 10% (5/52) pCR.

Gene polymorphisms in patients with wild-type KRAS

When gene polymorphism and tumor response were analyzed in the 59 patients with wild-type KRAS, EGF 61 remains to be significantly associated with the pCR, with the EGF G/G genotype had a pCR of 40% (2/5), compared with 12% (3/25) in patients heterozygous, and 0% (0/24) in patients homozygous for the A/A allele ($P = 0.019$, Fisher's exact test). On the other hand, TS 5'UTR polymorphism was no longer significantly associated with pCR ($P = 0.67$, data not shown).

Analysis of other tested germline polymorphisms

We did not observe statistically significant associations between other tested gene polymorphisms and pCR (Table 3).

EGF A61G SNP was the only polymorphism predicted pCR, which was validated by the leave-one-out cross validation method (118 of 121 leave-one-out datasets) through all the steps of analyses including the univariate, multivariate analysis, and subgroup analysis among KRAS wild-type patients.

Table 3. Genomic polymorphisms and tumor response in patients with rectal cancer treated with neoadjuvant chemoradiation

Polymorphism	N ^c	pCR		Dworak response			
		Yes	No	1	2	3	4
IL-8 T-251A							
TT	38	8 (22%)	28	7	12	9	8
TA	62	7 (12%)	53	9	29	15	7
AA	26	4 (16%)	21	4	12	5	4
P ^a		0.37 (0.74)		0.54 (0.70)			
MTHFR C677T							
CC	59	12 (20%)	47	9	23	15	12
CT	56	4 (8%)	48	9	27	12	4
TT	11	3 (30%)	7	2	3	2	3
P ^a		0.070 (0.37)		0.50 (0.70)			
MTHFR A-1298C							
AA	54	8 (16%)	43	7	26	10	8
CA	62	8 (13%)	52	12	25	15	8
CC	8	3 (38%)	5	0	2	3	3
P ^a		0.18 (0.72)		0.30 (0.70)			
FCGR2A H131R							
HH	35	4 (12%)	30	6	13	11	4
HR	69	11 (17%)	55	11	31	13	11
RR	19	4 (22%)	14	2	9	3	4
P ^a		0.61 (0.85)		0.77 (0.82)			
FCGR3A V158F							
VV	49	6 (12%)	43	7	23	13	6
VF	37	3 (9%)	32	8	15	9	3
FF	15	3 (20%)	12	3	8	1	3
P ^a		0.49 (0.85)		0.55 (0.70)			
CYCLIND1 A870G							
AA	32	5 (17%)	25	6	15	4	5
AG	58	7 (12%)	49	9	23	17	7
GG	34	6 (18%)	27	5	15	7	6
P ^a		0.76 (0.85)		0.55 (0.70)			
XRCC3 Thr241Met							
TT	51	6 (12%)	42	11	21	10	6
CT	55	9 (17%)	45	7	26	12	9
CC	12	2 (18%)	9	0	4	5	2
P ^a		0.74 (0.85)		0.059 (0.24)			
VEGF C936T							
CC	93	13 (15%)	75	16	40	19	13
CT ^b	30	6 (19%)	26	4	13	9	6
TT ^b	2						
P ^a		0.58 (0.85)		0.33 (0.70)			
EGFR G497A							
GG	71	10 (15%)	57	13	24	20	10
AG	42	8 (19%)	34	5	23	6	8
AA	12	1 (9%)	10	2	6	2	1
P ^a		0.75 (0.85)		0.61 (0.70)			
EGFR (CA) _n repeat							
Both <20	58	10 (18%)	47	9	23	15	10
Any ≥20	47	9 (21%)	34	5	20	9	9
P ^a		0.80 (0.85)		0.83 (0.83)			

(Continued on the following page)

Table 3. Genomic polymorphisms and tumor response in patients with rectal cancer treated with neoadjuvant chemoradiation (Cont'd)

Polymorphism	N ^c	pCR		Dworak response			
		Yes	No	1	2	3	4
EGF A61G							
AA	54	1 (2%)	49	14	24	11	1
AG	53	11 (21%)	41	4	25	12	11
GG	11	5 (45%)	6	2	1	3	5
P ^a		<0.001 (0.002)			<0.001 (<0.001)		
TS 3' UTR							
+/+	55	8 (15%)	44	7	21	16	8
+/-	56	9 (16%)	46	10	25	11	9
P ^a -/-	14	2 (15%)	11	3	7	1	2
		1.00 (1.00)			0.24 (0.70)		
TS 5' UTR							
2R/2R	15	5 (36%)	9	3	1	5	5
2R/3R	56	9 (16%)	46	11	23	12	9
3R/3R	24	0 (0%)	23	4	14	5	0
P ^a		0.006 (0.045)			0.015 (0.12)		
COX-2 G-765C							
GG	84	15 (19%)	66	12	37	17	15
GC ^b	38	4 (10%)	35	8	16	11	4
CC ^b	3						
P ^a		0.30 (0.72)			0.47 (0.70)		
RAD G51C							
GG	102	14 (14%)	84	16	43	25	14
GC	21	5 (25%)	15	3	9	3	5
P ^a		0.31 (0.72)			0.61 (0.70)		
KRAS let-7 lcs6 poly							
TT	109	15 (14%)	92	20	48	24	15
TG ^b	14	4 (29%)	10	0	5	5	4
GG ^b	3						
P ^a		0.23 (0.72)			0.024 (0.13)		

^aNominal *P* values were based on Fisher's exact test for pCR and exact conditional test for Dworak response. FDR-adjusted *P* values were shown in parentheses.

^bCombined in the analysis.

^cNumber of patients by each genotype. Some of the patients had response not evaluable.

Discussion

With the development of the total mesorectal excision technique and the current neoadjuvant chemoradiation regimen, the local recurrence rate in locally advanced rectal cancer patients has decreased significantly from 20% to 30% to less than 10%, and is no longer the reason for poor survival in these patients (19). Interestingly, this drop in local recurrence has not been correlated with better survival, and the high rate of distant metastasis appeared to be the problem for this failure (20). Therefore, targeted therapies have been added to the standard care of neoadjuvant chemoradiation for better local control and survival. Complete pCR has been shown to be strongly associated with the effectiveness of treatments and has been used to predict

survival following neoadjuvant therapy (21). However, recent phase I/II clinical trials with cetuximab-based chemoradiation therapy have failed to show improved pCR or survival in locally advanced rectal cancer patients. Question has been raised whether a subset of patients might benefit from this treatment. Our study suggests EGF A+61G polymorphism is significantly associated with pCR to cetuximab-based neoadjuvant chemoradiation in locally advanced rectal cancer patients, independent of KRAS mutation status. This is the first study suggesting this polymorphism might be a predictive marker for response to cetuximab-based therapy.

The EGF/EGFR pathway plays important roles in the pathogenesis and progression of human cancers (22) and both EGF and EGFR are commonly overexpressed in

Table 4. Logistic regression model of EGF A61G and TS 5' UTR polymorphisms and pCR

Polymorphism	Univariate analysis OR (95% CI)	Multivariate analysis ^b OR (95% CI)
EGF A61G		
AA	1 (reference)	1 (reference)
AG or GG	16.68 (95% CI: 2.13–130.82)	13.08 (95% CI: 1.51–113.54)
<i>P</i> ^a	0.007	0.020
TS 5' UTR		
2R/2R	1 (reference)	1 (reference)
2R/3R or 3R/3R	0.24 (95% CI: 0.06–0.86)	0.30 (95% CI: 0.06–1.52)
<i>P</i> ^a	0.028	0.14

^aBased on Wald test.^bStratified by study center and age (<60 vs. 60+ years old).

CRC. Epidermal growth factor (EGF) is encoded by a single gene on chromosome 4q25–q27. In 2002, Shahbazi and colleagues identified a G to A polymorphism at position 61 in the 5' untranslated region of EGF that is significantly correlated with production of EGF (23). Individuals with the G/G or A/G genotypes had higher EGF production *in vitro* than individuals with the A/A genotype, and homozygosity of the G allele was shown to be associated with the risk of developing malignant melanoma and several other malignancies, including gastric cancer (24), glioma (25), hepatocellular carcinoma in cirrhotic patients (26), CRC, and recurrence of liver metastasis from CRC (27), although subsequent studies showed conflicting results (28–31). On the other hand, several recent studies have shown that the G/G genotype is associated with improved OS and progression-free survival (PFS) or less tumor recurrence in patients with metastatic colorectal and esophageal cancers (32–34). Graziano and colleagues investigated 110 metastatic CRC patients who were treated with cetuximab-irinotecan salvage therapy and found significant association between EGF 61 G/G genotype and a favorable OS in these patients. In another group of 130 mCRC patients who were treated with single agent cetuximab, EGF + 61 G/G genotype was associated with better PFS (35). These apparently paradoxical results are possibly due to the different effect of increased EGF activity in different stages of cancer development. Overproduction of the EGF can impose an increased risk to develop cancer on normal cells, however, it might also increase cancer cells' susceptibility to treatment, especially targeted therapy such as cetuximab. Indeed, in our study, EGF61 G allele is strongly associated with pCR in this group of locally advanced rectal patients treated with cetuximab-based neoadjuvant chemoradiation. Our data are in line with the result of Khambata-Ford and colleagues that mCRC patients whose tumors with increased expression of EGF ligands epiregulin and amphiregulin are more likely to have disease control and PFS when treated with cetuximab (36). Whether this association of EGF 61 polymorphism and pCR is due to enhanced inhibi-

tion of the EGFR pathway or increased sensitivity to radiation therapy requires further investigation. KRAS mutation status has been shown to predict response to cetuximab-based therapy in mCRC patients (37) and mCRC patients with mutant-type KRAS are more likely to have a worse response, PFS, and OS when treated with cetuximab (38). On the other hand, studies of cetuximab-based neoadjuvant therapy in rectal cancer patients are few and have generated inconclusive results about the significance of KRAS mutation status and response to cetuximab therapy (39), likely due to small patient numbers of these studies. Our data showed that the KRAS mutation status is not statically significantly associated with the pCR. However, only 10 of 101 patients with known KRAS mutation status had pCR to cetuximab-based neoadjuvant chemoradiation therapy. This small sample size limited our ability to interpret the result and larger trials are required to make the conclusion. However, our data showed that the EGF61 A>G polymorphism has significant association with pCR to the cetuximab-based therapy in patients with both KRAS wild-type and mutant tumors.

Thymidylate synthase catalyze the reaction from dUMP to dTMP, the only *de novo* source of dTMP in cells, and is the therapeutic target for 5FU and its derivatives. High TS expression levels and activity have been associated with poor prognosis and response rates in CRC patients (40). The variable number of tandem repeats (VNTR) of a 28-bp sequence (2R and 3R) located within the 5' untranslated region (5'UTR) is one of the 3 polymorphisms identified to affect the expression of TS gene, located at 18p11.32. Studies have shown that the wild-type 3R enhance TS protein translation and elevates enzyme activity (41). However, data on the prognostic and predictive value of the VNTR has been mixed and conflicting. TS 5' UTR 3R genotype was linked to lower response rate (42), better survival outcome (43), better response to chemotherapy, or no significant difference in outcome (44) in advanced CRC patients treated with 5FU. On the

other hand, heterozygous 2R/3R genotype was associated with worse overall survival in one study (45) and was a favorable marker in others (46). Possible explanations for these inconsistent results include the multiple polymorphisms existing in the TS gene including the G/C SNP in the 3G allele (47), the 6bp deletion within the 3'UTR (48), and variations of TS gene copy number due to loss of heterozygosity (49) or gene amplification. In our study, TS 5'UTR 3R/3R genotype was significantly associated with the worst response rate (0% pCR) to cetuximab-based neoadjuvant chemoradiation therapy, compared with 33% pCR in patients with 2R/2R genotype, and 16% pCR in patients with heterozygous 2R/3R genotype ($P = 0.006$). Interestingly, this association is not significant in patients with wild-type KRAS. This result might be confounded by the treatment of all patients with 5FU or capecitabine.

In conclusion, our study suggests intratumoral EGF61 A/G polymorphism to be a potential predictive marker for pCR to cetuximab-based neoadjuvant chemoradiation in patients with locally advanced rectal cancer. The retrospective nature and relatively small sample size limited the significance of this study. Furthermore, the patients all received cetuximab-based therapy and there is no untreated control group. Therefore the results of this study should be

interpreted carefully and validated in larger prospective trials.

Disclosure of Potential Conflicts of Interest

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