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Epidermal growth factor receptor R521K polymorphism shows favorable outcomes in *KRAS* wild-type colorectal cancer patients treated with cetuximab-based chemotherapy

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The R521K polymorphism of epidermal growth factor receptor has attenuated affinity in ligand binding and proto-oncogene induction, which may affect the efficacy of cetuximab. We analyzed the effect of this polymorphism on the outcome of 112 patients with KRAS wild-type metastatic colorectal carcinoma treated with first-line cetuximab plus FOLFOX-4. The associations of this polymorphism with vascular endothelial growth factor (VEGF) expression and clinicopathologic characteristics were also examined. The results showed that the frequencies of the G/G, G/A, and A/A genotypes were 32.1% ($n = 36$), 42.9% ($n = 48$), and 25.0% ($n = 28$), respectively. A marked decrease in VEGF expression levels (66.7% *vs* 28.9%, $P < 0.01$) was observed in patients with 521A allele variants (Arg/Lys or Lys/Lys), which were associated with a decreased tumor size (55.6% *vs* 31.6%, $P = 0.02$), good histological differentiation (63.9% *vs* 85.5%, $P = 0.01$), decreased lymphovascular invasion (69.4% *vs* 39.5%, $P < 0.01$), and a higher response rate to cetuximab plus FOLFOX treatment (55.6% *vs* 78.9%, $P = 0.01$). In addition, this polymorphism was associated with a longer progression-free period ($P = 0.001$) and overall survival ($P = 0.001$). By multivariate analysis, this polymorphism was also identified as an independent prognostic factor. These data suggest that the R521K polymorphism of epidermal growth factor receptor, by reducing its activation and a consequential downregulation of its target genes, including VEGF, could be a key determinant of an increased response to cetuximab-based chemotherapy and a longer survival for *KRAS* wild-type colorectal carcinoma patients. (*Cancer Sci* 2012; 103: 791–796)

Cetuximab, a chimeric mAb, is an antibody against the extracellular domain of epidermal growth factor receptor (EGFR).[1] It binds to EGFR with a high affinity and is able to compete with epidermal growth factor (EGF) binding, thereby inhibiting subsequent receptor activation and signalling.[1] Cetuximab is approved for the treatment of patients with metastatic colorectal carcinoma (CRC) and squamous carcinoma of the head and neck. A favorable effect of cetuximab combined with chemotherapy in advanced non-small-cell lung cancer (NSCLC) has also been reported.[2] The benefits of cetuximab-based therapies are restricted to a particular subgroup of patients. The EGFR expression, as evaluated by immunohistochemistry, does not correlate with the response to cetuximab,[3] but an increase in *EGFR* copy number identified by FISH, may predict a better response.[4] Loss of phosphatase and tensin homolog (PTEN) protein expression is also associated with a lower response rate.[5]

Mutations of *KRAS*, a gene encoding a G protein that plays a key role in the downstream signaling of EGFR, lead to resistance to cetuximab.[6] Mutations of *KRAS* occur in approximately 40% of CRC, and the mutation status of *KRAS* is considered a good predictive marker for cetuximab-based treatment.[6] Mutations of the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) and *PIK3CA* genes are also associated with a lower response rate to cetuximab, and the prediction of response rates could be improved by additional genotyping of these genes.[7]

In *KRAS* wild-type populations, there remains a subgroup of patients who do not respond to cetuximab; therefore, additional markers for predicting the response are indicated. Genetic variations in *EGFR* or its ligand, EGF, may predict clinical outcome in patients treated with cetuximab-based regimens.[8,9] For example, the A61G polymorphism of *EGF* predicts the effect of cetuximab plus irinotecan.[8] In addition, the intron 1 CA dinucleotide repeat polymorphism of *EGFR* predicts survival of *KRAS* wild-type patients treated with cetuximab-based chemotherapy.[9]

A polymorphic variant in *EGFR* arising from a single nucleotide substitution (142285 G>A), leading to an arginine (R)/lysine (K) substitution in codon 521 in the extracellular domain of EGFR, has been identified.[10] This polymorphism, previously described as R497K according to an older nomenclature, was found to be associated with a lower pelvic recurrence in rectal cancer patients treated with chemoradiation,[11] longer survival in stage II/III CRC patients who received curative surgery, and a better response to oxaliplatin-based chemotherapy.[12] Compared with the wild-type 521R allele, the 521K allele variant has attenuated affinity in ligand binding, tyrosine kinase activation, and induction of the proto-oncogenes *myc*, *fos*, and *jun*.[13] In breast cancer patients, the 521K allele variant was found to be associated with decreased lymph node metastasis and good histological differentiation.[14] Interestingly, this polymorphism was associated with progression-free survival in CRC patients treated with single-agent cetuximab.[15] In a retrospective study of 32 CRC patients with different *KRAS* status treated with cetuximab plus irinotecan, this polymorphism was found to be associated with higher response rate and longer survival; however, the enrolled patients were quite heterogeneous.[4]

Based on these earlier findings, we propose that the R521K polymorphism of EGFR might be associated with a better outcome in CRC patients receiving cetuximab-based treatments. A study was carried out in 112 *KRAS* wild-type CRC patients treated with first-line cetuximab plus FOLFOX-4, and the

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potential of using this polymorphism as a predictive/prognostic marker was evaluated.

Materials and Methods

Patient characteristics. We examined 118 consecutive patients with *KRAS* wild-type metastatic CRC who had received cetuximab plus FOLFOX-4 as a first-line treatment, from January 2006 to December 2009. Among them, 112 patients were enrolled and analyzed. The remaining patients were excluded; these patients lacked measurable lesions ($n = 3$), did not have the primary tumor removed to examine the accurate T and N stages ($n = 2$), or were unwilling to participate ($n = 1$). Patients were treated with cetuximab (500 mg/m^2, 2-h infusion, day 1; Merck Serono, Darmstadt, Germany), followed by FOXFOX-4. The FOLFOX-4 regimen consisted of oxaliplatin (85 mg/m^2, 1-h infusion, day 1; Sanofi-Aventis, Paris, France) and folinic acid (200 mg/m^2, 2-h infusion, days 1 and 2) before bolus 5-fluorouracil (5-FU; 400 mg/m^2, days 1 and 2; Pharmachemie, Ga Haarlem, Holland), and infusional 5-FU (600 mg/m^2, 22-h infusion immediately after bolus 5-FU, days 1 and 2) given every 2 weeks.

The response to treatment was evaluated on the basis of standard RECIST criteria. In the case of intolerable toxicity or failure to respond to front-line cetuximab plus FOLFOX-4, the treatment was discontinued, and irinotecan-based or fluoropyrimidine-only regimens were begun according to the physicians' decision. During treatment, chest X-ray and CT scan of the abdomen were carried out every 2 months. None of the patients was treated with antagonists that would interfere with the vascular endothelial growth factor (VEGF) pathway. All patients were followed up until disease progression, death, or loss to follow-up at a similar intensity regardless of *EGFR* polymorphism status. Patients with different *EGFR* genotypes were followed up with a median duration of 20 months (range, 5–32 months). An institutional review board approved this study and informed consent was received from all patients before blood testing for genotyping.

Examination of *KRAS* mutations. Exon 1 of *KRAS*, the most frequent site of activating mutations in codons 12 and 13, was sequenced after PCR amplification according to a method previously described.[16] DNA was extracted from patients' tumor tissue, including frozen ($n = 46$) or formalin-fixed paraffin-embedded samples ($n = 66$), using standard phenol–chloroform procedures. Adjacent sections were stained with H&E to confirm the presence of at least 50% carcinoma tissue at this location. Briefly, 0.1 μg genomic DNA, forward primer 5′-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3′ and reverse primer 5′-TCA AAG AAT GGT CCT GGA CC-3′, were used for PCR amplification. The PCR cycle conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 96°C for 60 s, 55°C for 60 s, 73°C for 30 s, and a final elongation at 72°C for 10 min. A negative (no DNA) control was run with each PCR analysis. After amplification, the PCR products were sequenced directly.

Examination of the R521K polymorphism of EGFR. Genomic DNA was extracted from patients' WBCs obtained from 0.5 mL whole blood using standard phenol–chloroform procedures. The R521K polymorphism of EGFR was examined by the PCR-RFLP method as previously described.[17] Briefly, 0.1 μg genomic DNA, forward primer 5′-TGC TGT GAC CCA CTC TGT CT-3′ and reverse primer 5′-CAA CGC AAG GGG ATT AAA GA-3′ were used for PCR amplification. The PCR cycle conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 30 s, and a final elongation at 72°C for 10 min. The PCR products, after being digested by the *Sty*I restriction enzyme (New England Biolabs, Beverly, MA, USA) at 37°C for 16 h, were separated on 3% ethidium bromide-stained agarose gels. The RFLP data were compared with PCR-direct sequencing results to avoid genotyping errors.

Examination of genetic polymorphisms involved in nucleotide excision repair (NER) pathway and expression of the thymidylate synthase gene. Because polymorphisms of genes that are involved in the NER pathway, including excision repair cross-complementing group 1 (ERCC1) and xeroderma pigmentosum group D (XPD), contribute to resistance to FOLFOX-4 treatment,[18,19] the influence of polymorphisms involved in the NER pathway warrants further study. Genomic DNA, extracted from patients' WBCs, was subjected to PCR amplification. *ERCC1* codon 118 C→T and *XPD* K751Q polymorphisms were examined by the PCR-RFLP method as previously described.[18,19] The PCR products, after being digested with *Bsr*D1 (for *ERCC1*) or *Mbo*II (for *XPD*) restriction enzymes (New England Biolabs), were separated on Nusieve ethidium bromide-stained agarose gels (Lonza, Basel, Switzerland) to determine different genotypes.

As 5-FU was used in combination with oxaliplatin to treat these patients, and germ-line polymorphisms of a 28-bp tandemly repeated sequence in the 5′-enhancer region of the thymidylate synthase gene (*TSER*) remarkably affect the response and survival of CRC patients receiving 5-FU,[20] the influence of this polymorphism on patients with different *TSER* genotypes warranted further analysis. The polymorphism of *TSER* was examined by PCR method as previously described.[20] The amplified DNA fragments were analyzed by electrophoresis on a 4% agarose gel to determine the number of 28-bp tandemly repeated sequences in *TSER*.

Immunohistochemistry. Because EGFR signaling regulates the synthesis of several pro-angiogenic growth factors, including VEGF,[21] we propose that the R521K polymorphism of EGFR, by attenuating its ligand binding and subsequent activation of downstream effectors, may be associated with reduced expression of VEGF. Paraffin-embedded tumor tissue sections were stained with an anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), using a streptavidin–biotin immunoperoxidase kit (BioGenex, San Ramon, CA, USA), according to the manufacturer's instructions. Two independent pathologists examined these slides microscopically, and the staining result of VEGF was divided into two groups according to the percentage of carcinoma cells showing specific IHC signals: less than 10%, and more than 10% positive cells.

Statistical analysis and survival curve plotting. Patients were divided into "G/G (wild-type)" and "G/A or A/A (variant-type)" groups according to the different *EGFR* codon 521 genotypes. Cause-specific survival curves were plotted using the Kaplan–Meier product limit method, and the statistical differences in survival between the subgroups were compared using the log–rank test. The correlations of VEGF expression levels, tumor size, histological differentiation, lymphovascular invasion, genetic polymorphisms involved in the NER pathway, and response to cetuximab plus FOLFOX treatment, were analyzed separately according to the codon 521 status of *EGFR*. The statistical differences in these correlations were determined using the chi squared-test. To assess the independent prognostic value of this polymorphism, we used Cox's proportional hazards regression analysis (multivariate), which included *EGFR* codon 521 status and other clinicopathologic parameters. All statistical analyses were carried out using SPSS for Windows (version 10.0; SPSS, Chicago, IL, USA).

Results

R521K polymorphism of EGFR correlates with reduced VEGF expression levels. An example of different *KRAS* mutation patterns analyzed by the PCR-direct sequencing method is

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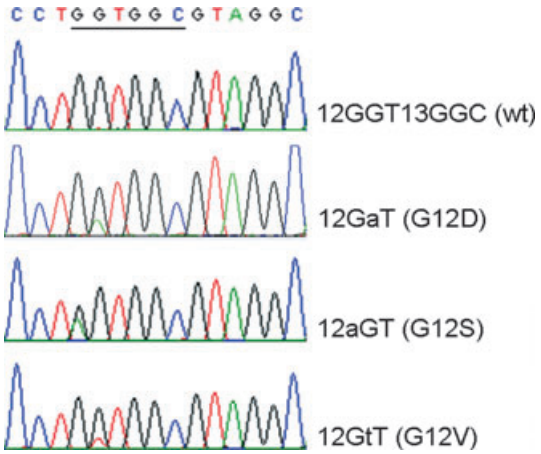


Fig. 1. Representative patterns of different *KRAS* exon 1 genotypes. The PCR products containing codons 12 and 13 of *KRAS* were amplified with PCR. After amplification, the PCR products were sequenced directly. wt, wild type.

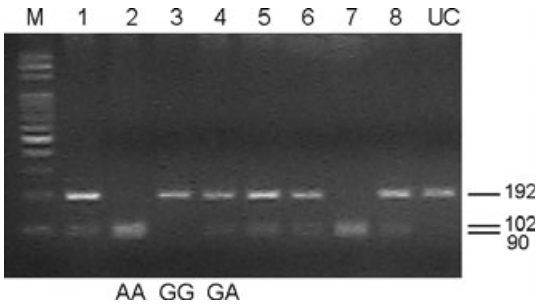


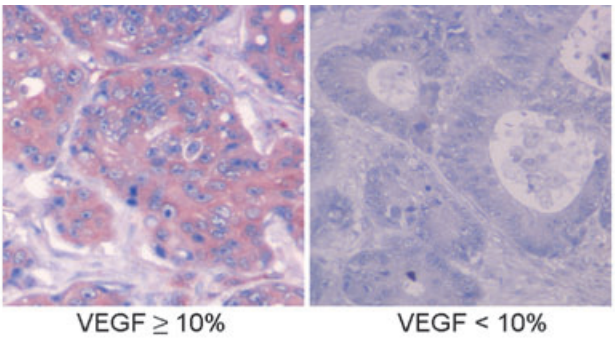
Fig. 2. Representative PCR-RFLP patterns of different *EGFR* 521 genotypes. Genomic DNA obtained from patients' WBCs was subjected to PCR amplification. After being digested by *Sty*I, the PCR products were separated by agarose gel electrophoresis. Lanes 1, 4, 5, 6, and 8 represent G/A; lanes 2 and 7 represent A/A; lane 3 represents G/G. M, marker; UC, PCR product that has not been digested.

shown in Figure 1. Different allele patterns of the R521K polymorphism of *EGFR* analyzed by the PCR-RFLP method are shown in Figure 2. The frequencies of *EGFR* codon 521 G/G (wild-type), G/A, and A/A genotypes were 32.1% (n = 36), 42.9% (n = 48), and 25.0% (n = 28), respectively. As shown in Figure 3, a marked decrease in VEGF expression was observed in patients with the R521K polymorphism, as the percentage of patients with a higher VEGF expression level (more than 10% positive cells) in those with or without the R521K polymorphism was 28.9% and 66.7%, respectively (P < 0.01; Fig. 3).

R521K polymorphism of EGFR associated with decreased tumor size, good histological differentiation, and lower rate of lymphovascular invasion. As the R521K polymorphism of EGFR dramatically decreases the activation of one of its downstream effectors, VEGF, alterations in clinicopathologic features and the prognosis of patients carrying this polymorphism were proposed. Therefore, the correlation between this polymorphism and the clinicopathologic characteristics of these patients were examined. As shown in Table 1, decreased tumor size (P = 0.02), good histological differentiation (P = 0.01), and a lower rate of lymphovascular invasion (P < 0.01), were clearly identified in patients with the R521K polymorphism. However, there were no between-group differences in age, gender, performance status, or location of the primary tumor of patients with different *EGFR* codon 521 statuses (Table 1).

R521K polymorphism of EGFR correlates with a higher response rate to cetuximab plus FOLFOX and a favorable prognosis in *KRAS* wild-type CRC patients. Preliminary studies have shown that the R497K (or R521K) polymorphism of EGFR is predictive of FOLFOX-4[12] and cetuximab/irinotecan combination treatment benefit,[4] so we speculated whether *KRAS* wild-type CRC patients with this polymorphism might be more sensitive to cetuximab-based treatment, which could translate into a favorable prognosis. As shown in Table 2, patients with G/A or A/A genotypes have a significantly higher response rate to treatment (78.9% *vs* 55.6%, P = 0.01), longer progression-free period (8 *vs* 16 months, P < 0.01; Fig. 4A), and overall survival (16 *vs* 24 months, P < 0.01; Fig. 4B). With adjusted analysis, this polymorphism was identified as an independent prognostic factor (P = 0.02; Table 3). Second-line chemotherapy also affects the survival of patients with metastatic CRC. In the current study, a subset of the patients (n = 96) was treated with second-line chemotherapy, and improved survival was clearly shown (P = 0.02; Table 3).

Although polymorphisms of the *ERCC1* and *XPD* genes had a significant impact on survival of patients treated with cetuximab plus FOLFOX in this study (P < 0.01; Table 3), there were no between-group differences in these genetic alterations among patients with different *EGFR* codon 521 genotypes (Table 1). Therefore, the influences of these polymorphisms in different *EGFR* genotypes could be neglected.



	VEGF ≥ 10%	VEGF < 10%	*P*
521 G/G	24 (66.7%)	12 (33.3%)	<0.01
521 G/A or A/A	22 (28.9%)	54 (71.1%)	

Fig. 3. Representative immunohistochemical staining patterns of vascular endothelial growth factor (VEGF). The staining results for VEGF were divided into two groups, <10% and more than 10% positive cells, according to the percentage of carcinoma cells showing specific immune-reactivity.

Discussion

Somatic mutations of *EGFR* are associated with increased sensitivity to EGFR-tyrosine kinase inhibitors in NSCLC,[22] but such mutations are rare or absent in CRC,[23] and the addition of gefitinib to chemotherapy does not improve therapeutic efficacy in CRC patients.[24] *KRAS* mutations lead to resistance to cetuximab;[6] but in *KRAS* wild-type populations, there remains a subgroup of patients that does not respond to cetuximab. Polymorphisms in *EGFR* have attracted a lot of attention because they affect not only their function in ligand binding,[13] but also clinicopathologic features[14] and response to current therapeutic agents,[11,12] including cetuximab.[4,15] In the current study, we focused on the extracellular domain of EGFR because this region has been shown to be highly polymorphic and to have a significant impact on the function in ligand binding and subsequent signaling.[13] The R521K variant has also been described as being associated with cancer severity in EGFR-expressing tumors, such as gliomas and lung cancer.[25]

Table 1. Clinicopathologic features according to epidermal growth factor receptor R521K polymorphism status in *KRAS* wild-type colorectal carcinoma patients (*n* = 112)

Characteristics	G/G (wild-type) (%)	G/A or A/A (%)	*P*
All patients	36 (100)	76 (100)	
Age, years			
<50	14 (38.9)	26 (34.2)	0.63
≥50	22 (61.1)	50 (65.8)	
Gender			
Male	25 (69.4)	55 (72.4)	0.75
Female	11 (30.6)	21 (27.6)	
Performance status			
0	26 (72.2)	53 (69.7)	0.79
1, 2	10 (27.8)	23 (30.3)	
Primary tumor			
Colon	24 (62.1)	57 (69.0)	0.36
Rectum	12 (37.9)	19 (31.0)	
VEGF expression			
Higher (≥10% positive cells)	24 (66.7)	22 (28.9)	<0.01
Lower (<10% positive cells)	12 (33.3)	54 (71.1)	
At least one tumor ≥6 cm			
Present	20 (55.6)	24 (31.6)	0.02
Absent	16 (44.4)	52 (68.4)	
Histologic differentiation			
Good/moderate	23 (63.9)	65 (85.5)	0.01
Poor	13 (36.1)	11 (14.5)	
Lymphovascular invasion			
Present	25 (69.4)	30 (39.5)	<0.01
Absent	11 (30.6)	46 (60.5)	
ERCC1 codon 118 genotype			
C/C (wild-type)	17 (47.2)	35 (46.1)	0.91
C/T or T/T	19 (52.8)	41 (53.9)	
XPD codon 751 status			
Lys/Lys (wild-type)	31 (86.1)	63 (82.9)	0.67
Lys/Gln	5 (13.9)	13 (17.1)	
TSER 28-bp polymorphism			
2R/2R or 2R/3R	12 (33.3)	27 (35.5)	0.82
3R/3R	24 (66.7)	49 (64.5)	

bp, base pair; ERCC1, excision repair cross-complementing group 1; TSER, 5′-enhancer region of the thymidylate synthase gene; VEGF, vascular endothelial growth factor; XPD, xeroderma pigmentosum group D.

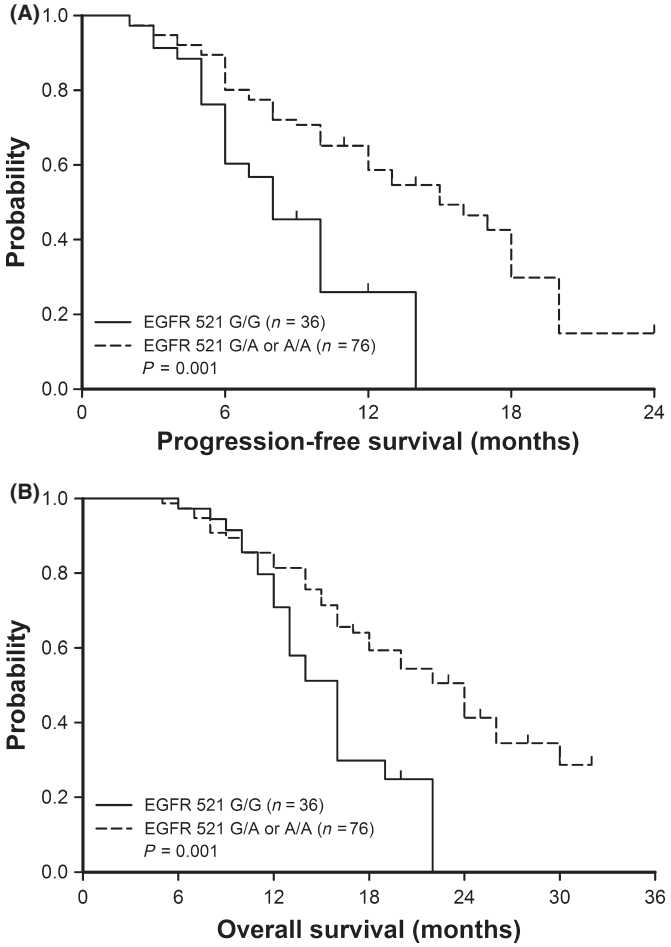


Fig. 4. Epidermal growth factor receptor (*EGFR*) codon 521 G/A or A/A genotypes in *KRAS* wild-type metastatic colorectal carcinoma patients are associated with longer progression-free as well as overall survival. (A) Progression-free survival curves of 112 metastatic colorectal carcinoma patients with different *EGFR* codon 521 genotypes were plotted by the Kaplan–Meier method ($P = 0.001$; log–rank test). (B) A similar method was used to plot overall survival curves ($P = 0.001$; log–rank test).

Table 2. Response rate to cetuximab plus FOLFOX-4 in *KRAS* wild-type colorectal carcinoma patients with different epidermal growth factor receptor codon 521 statuses (*n* = 112)

Response	G/G (wild-type) (%)	G/A or A/A (%)	*P*
All patients enrolled	36 (100)	76 (100)	
OR (CR + PR)	20 (55.6)	60 (78.9)	0.01
CR	2 (5.6)	3 (3.9)	
PR	18 (50.0)	57 (75.0)	
SD	9 (25.0)	11 (14.5)	
PD	7 (19.4)	5 (6.6)	

Comparison of overall response rate between patients with different EGFR R521K polymorphisms. CR, complete remission; OR, overall response; PD, progressive disease; PR, partial remission; SD, stable disease.

In a retrospective study of 32 patients with metastatic CRC treated with cetuximab plus irinotecan, the R521K polymorphism of EGFR was found to be associated with higher response rate.[4] However, the enrolled patients were quite heterogeneous. In 22 (68.8%) patients, cetuximab/irinotecan was given after two or more regimens failed, and a variety of previous chemotherapy was used in different individuals. One of them was even chemotherapy-naive. In addition, the enrolled patients had different *KRAS* statuses, including 14 (43.8%) patients with *KRAS* mutations and another 18 with wild-type *KRAS,* making difficult to ascertain the predictive/prognostic value of this EGFR polymorphism in this study.

The R521K (or R497K according to an older nomenclature) polymorphism of EGFR was previously found to be a prognostic marker for CRC patients because it was associated with longer survival in stage II/III CRC patients treated with curative surgery.[12] In the current study, we further identified that this polymorphism could serve as a predictive marker for cetuximab-based treatment in *KRAS* wild-type CRC. We found that the EGFR R521K polymorphism correlates with a higher response rate (Table 2) and a favorable prognosis (Fig. 4). An attenuated EGFR signaling induced by the R521K polymorphism that could be more sensitive to targeted receptor inhibition was proposed. The resulting amino acid substitution, arginine to lysine, has been shown to reduce ligand binding

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Table 3. Analysis of factors that may affect the survival of patients with *KRAS* wild-type colorectal carcinoma (n = 112)

Characteristics	P (univariate)	P (multivariate)	HR
Age (years)			
<50 *vs* ≥50	0.48	NA	NA
Gender			
Male *vs* female	0.36	NA	NA
Performance status			
0 *vs* 1, 2	0.21	NA	NA
Primary tumor			
Colon *vs* rectum	0.45	NA	NA
VEGF expression level			
≥10% *vs* <10% positive cells	0.02	0.06	1.68
At least one tumor ≥6 cm			
Presence *vs* absence	0.15	NA	NA
Histologic differentiation			
Good–moderate *vs* poor	0.13	NA	NA
Lymphovascular invasion			
Presence *vs* absence	0.06	NA	NA
Metastasis at diagnosis			
Yes *vs* No	0.03	<0.01	3.82
Serum CEA level (ng/mL)			
≤6 *vs* >6	0.34	NA	NA
Second-line chemotherapy			
No *vs* yes	0.03	0.02	2.32
EGFR R521K polymorphism			
G/G (wild-type) *vs* G/A or A/A	0.03	0.02	2.41
ERCC1 codon 118 genotype			
C/T or T/T *vs* C/C	0.01	<0.01	4.51
XPD codon 751 status			
Lys/Gln *vs* Lys/Lys	0.02	<0.01	3.35
TSER 28-bp polymorphism			
2R/2R+2R/3R *vs* 3R/3R	0.18	NA	NA

bp, base pair; CEA, carcinoembryonic antigen; EGFR, epidermal growth factor receptor; ERCC1, excision repair cross-complementing group 1; HR, Cox hazard ratio; NA, not analyzed; TSER, 5′-enhancer region of the thymidylate synthase gene; VEGF, vascular endothelial growth factor; XPD, xeroderma pigmentosum group D.

and ligand-induced EGFR signaling.[13] Therefore, this genetic variant may alter the binding of its specific ligands, leading to an altered phenotype of EGFR signaling. Of particular interest, the expression of EGFR ligands epiregulin and amphiregulin, which may predict cetuximab benefit, has also been clearly shown.[26] In fact, the resulting amino acid substitution is located at the boundary between EGFR domain III, which represents the direct interaction site with cetuximab, and domain IV.[27] Therefore, the 521K variant could affect cetuximab binding and/or effects.

In the current study, G/A or A/A genotypes in the CRC patients were associated with smaller tumor size, better histologic differentiation, and lower rate of lymphovascular invasion (Table 1). These data are compatible with a previous report that the R521K polymorphism is correlated with a lower tumor grade and fewer lymph node metastases, and might be a prognostic factor in breast cancer patients.[14] The association between the EGFR R521K polymorphism and lymph node metastasis deserves further consideration as a clinical indicator during pre-operative evaluation. In fact, various studies have considered genetic factors involving cell mobility, vascular invasion and angiogenesis for predicting lymph node metastasis.[28]

We found that the R521K polymorphism of EGFR correlates with a higher response rate to cetuximab-based treatment and a favorable prognosis in *KRAS* wild-type CRC patients (Table 2, Fig. 4). However, patients with this polymorphism also have lower VEGF expression levels (Fig. 3); therefore, the confounding bias between EGFR polymorphism and VEGF status should be considered. Epidermal growth factor receptor signaling regulates the synthesis of several pro-angiogenic growth factors, including VEGF.[21] Previous studies have shown that the expression of VEGF is related to the extent of tumor vascularization and prognosis, and is predictive of resistance to chemotherapy.[29] Interestingly, in a subset of human tumors, VEGF may promote the malignant progression of tumor cells by directly acting on its receptors through an endothelial cell-independent pathway.[30,31] The expression of VEGF receptor 1 (VEGFR1) and VEGFR2 has been shown in CRC cells, and the activation of these receptors by VEGF leads to activation of the MAPK pathway and phenotypic changes in CRC cells.[30,31] Clinically, the addition of bevacizumab, a mAb directed against VEGF, to chemotherapy improves response rates and survival for patients with metastatic CRC.[32]

Discrepancies in the association between genotype of *EGFR* codon 521 and its impact on clinical outcome of anti-EGFR therapy exist in different types of malignancy. The R521K polymorphism was associated with a longer progression-free survival in CRC patients treated with cetuximab.[15] In patients with NSCLC treated with gefitinib, although sensitivity to treatment strongly depends on the EGFR mutation status,[33] the R521K polymorphism of EGFR was not associated with response rate.[17] Whether this is due to different drugs used for treatment, or simply due to different tumor types, deserves further study. In addition, because FOLFOX-4 was used in combination with cetuximab in the current study, it remains possible that the improvement in survival seen in patients with G/A and A/A genotypes is not a reflection of increased sensitivity to cetuximab, but rather that this group inherently has a higher sensitivity to FOLFOX-4, or a longer survival owing to decreased EGFR signaling.[12]

Compared with Caucasian populations,[4] a remarkably higher prevalence (67.9%) of the EGFR 521K allele variants was noted in our patients. Ethnic differences have a profound influence on the response and toxicity to chemotherapy in malignant diseases. For example, the UGT1A1*28 polymorphism is rare in Asian populations, which leads to a decreased risk of developing severe neutropenia after being treated with irinotecan.[34] Due to a higher prevalence of *EGFR* mutations, gefitinib is very effective in Asian patients with NSCLC.[33] In the current study, the frequency of *EGFR* codon 521 A allele variants was 67.9%, including 42.9% G/A and 25.0% A/A genotypes, which was significantly higher than that in Caucasian (37.5%) and Tunisian (39.2%) populations.[4,14] This result implied that Asian populations might have an attenuated EGFR ligand binding affinity and a better outcome to cetuximab-based treatment, which deserves further study.

The activation of EGFR initiates intracellular proliferation signaling results in proliferation and survival through the Ras/Raf/MEK/ERK or PI3K/PTEN/AKT pathways, respectively.[7] Therefore, alterations of the downstream effectors of the EGFR pathway may have influence in its signaling and the efficacy of EGFR-targeted therapies. Loss of *PTEN* protein expression,[5] and mutation of *BRAF* have been associated with lower efficacy of therapies directed against *EGFR*-activated pathways.[35] However, mutations in BRAF were identified in less than 5% of CRC patients,[35] and this mutation is not routinely examined in clinical practice. Mutation of the *PIK3CA* gene, resulting in a mutant PI3-kinase and constitutively activated Akt signaling,[36] is also associated with a lower response rate to cetuximab.[7] The prediction of response rates to cetuximab could be improved by additional genotyping of these genes,

and prospective studies in clinical trial cohorts will be required to confirm the utility of these markers.

In summary, we showed that the R521K polymorphism of EGFR is associated with decreased VEGF expression in CRC cells. By reducing the activation of EGFR and consequential downregulation of its target genes, this polymorphism is likely to be one determinant of increased response to cetuximab-based chemotherapy and longer survival for *KRAS* wild-type CRC patients.

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Disclosure Statement

The authors have no conflicts of interest.

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