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Polymorphisms in *VEGF* and *IL-8* predict tumor recurrence in stage III colon cancer

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Background: Identifying molecular markers for tumor recurrence is critical in successfully selecting patients with stage III colon cancer who are more likely to benefit from adjuvant chemotherapy. The present study analyzed a subset of 10 polymorphisms within eight genes involved in the tumor angiogenesis pathway and their impact on prognosis in stage III colon cancer patients treated with adjuvant chemotherapy.

Patients and methods: Blood samples were obtained from 125 patients with locally advanced colon cancer at University of Southern California medical facilities. DNA was extracted from peripheral blood and the genotypes were analyzed using PCR–restriction fragment length polymorphism and 5′-end [γ-^{33}P] ATP-labeled PCR protocols.

Results: Polymorphisms in *vascular endothelial growth factor (VEGF)* (C+936T; $P = 0.003$, log-rank test) and *interleukin-8 (IL-8)* (T−251A; $P = 0.04$, log-rank test) were independently associated with risk of recurrence in stage III colon cancer patients. In combined analysis, grouping alleles into favorable versus nonfavorable alleles, high expression variants of *VEGF* C+936T and *IL-8* T−251A were associated with a higher likelihood of developing tumor recurrence ($P < 0.001$).

Conclusion: High expression variants of *VEGF* C+936T and *IL-8* T−251A were associated with shorter time to tumor recurrence, indicating that the analysis of angiogenesis-related gene polymorphisms may help to identify patient subgroups at high risk for tumor recurrence.

Key words: angiogenesis, colon cancer, tumor recurrence, *VEGF*

introduction

Colorectal cancer (CRC) is the third most common cancer in the United States. In the year 2007, an estimated 153 000 new cases will be diagnosed and 52 000 people will die from this disease [1]. For patients who undergo successful surgery for CRC, additional chemotherapy is recommended in stage III disease. Adjuvant chemotherapy with FOLFOX reduces the relative rate of recurrence by 23% and the overall death rate by 31% and is the standard of care for Stage III CRC patients [2–4]. Nevertheless, tumor recurrence after curative resection continues to be a significant problem in the management of CRC.

Angiogenesis, the formation of new blood vessels from endothelial precursors, is a prerequisite for the growth and progression of solid malignancies. Gaining access to the host vascular system and the generation of a tumor blood supply are rate-limiting steps in tumor growth and progression [5]. Tumors start as avascular masses which can initially thrive on preexistent vasculature within the microenvironment. When a tumor grows beyond a size of approximately 2–3 mm, as a consequence, the tumor requires its own new and dedicated vasculature. The so-called angiogenic switch, the induction of tumor vasculature or switch to an angiogenic phenotype, is considered a hallmark of the malignant process and is required for tumor propagation and progression [5].

Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEFGR-2 are intimately involved in the regulation of tumor-associated angiogenesis and have been reported to be correlated with a poor prognosis in various human malignancies. Recently, interleukin-8 (IL-8) has been reported to play a major role in VEGF-independent tumor angiogenesis. Induction of IL-8 preserved the angiogenic response in HIF1-α-deficient colon cancer cells, suggesting that IL-8 mediates angiogenesis, independently of VEGF [6]. In the postgenomic era, the possibility of individualized cancer treatment is gaining wider acceptance, and numerous germline polymorphisms in genes involved in the angiogenesis pathway that influence differential enzyme function or expression have been identified. However, there are

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only a few clinical and potential molecular markers, which can predict tumor recurrence in stage III CRC patients. These include microsatellite instability and 18q deletions [7]. The identification of molecular pathways is critical in understanding the mechanisms of tumor relapse and therefore essential in the development of more effective adjuvant treatment strategies.

Recognizing the complexity of disease progression, we designed a retrospective study to evaluate whether 10 polymorphisms within eight genes involved in the angiogenesis pathway, alone or in combination, were associated with an increased likelihood of tumor recurrence in stage III CRC patients treated with adjuvant chemotherapy.

patients and methods

patients

One hundred and twenty-five patients with stage III CRC who were treated with 5-flourouracil-based adjuvant chemotherapy at the University of Southern California/Norris Comprehensive Cancer Center (USC/NCCC) or the Los Angeles County/University of Southern California Medical Center, between 1992 and 2007, were eligible for the present study. This study was conducted at the USC/NCCC and approved by the Institutional Review Board of the University of Southern California for Medical Sciences. Patient data were collected retrospectively through chart review. Informed consent was signed by all patients involved in the study. Detailed clinicopathologic characteristics are shown in Table 1.

genotyping

Whole blood was collected and genomic DNA was extracted using the QIAamp kit (Qiagen, Valencia, CA). The majority of the samples were tested using PCR–restriction fragment length polymorphism technique. Briefly, forward and reverse primers were used for PCR amplification, PCR products were digested by restriction enzymes (New England Biolabs, Ipswich, MA) and alleles were separated using a 4% NuSieve ethidium bromide-stained agarose gel. Forward and reverse primers, restriction enzymes and annealing temperatures are listed in Table 3. Samples were analyzed by direct sequencing, if no matching restriction enzyme could be found.

The dinucleotide polymorphisms (Table 3) were determined with 5′-end 33p γATP-labeled PCR protocol with a few modifications. In summary, DNA template, deoxyribonucleotide triphosphate (dNTP), 5′-end 33p γATP-labeled primer, unlabeled complementary primer, Taq polymerase (Perkin Elmer Inc., Waltham, MA) and PCR buffer were used together in a final PCR. The reaction was carried out and the reaction products were separated using a 6% denaturing polyacrylamid DNA sequencing gel, which then was vacuum blotted for 1 h at 80°C and exposed to an XAR film (Eastman-Kodak Co., Rochester, NY) overnight. The exact number of repeats was confirmed by direct sequencing.

statistical analysis

The primary end point in this study was time to tumor recurrence (TTR) in stage III CRC patients, which was defined as the time from the date of diagnosis of stage III CRC to the date of first recurrence, death or until last contact if the patient was free of any tumor recurrence at the time of last contact. If a patient had not recurred, then TTR was censored at the time of death or at the last follow-up. The associations of TTR with patient's clinicopathologic characteristics (age, sex, race, tumor grade, T-stage, N-stage and type of chemotherapy) were assessed using univariate survival analyses (log-rank test).

The association between each polymorphism and time to recurrence was examined using Kaplan–Meier curves and log-rank test. The distributions of polymorphisms across demographic characteristics were examined using Fisher's exact test. In the univariate survival analysis, the Pike estimate of relative risk and its associated 95% confidence interval (95% CI) was based on the log-rank test.

The Cox proportional hazards regression model with stratification factors (race, number of resected lymph nodes and type of adjuvant therapy) was fitted to reevaluate the association between polymorphisms and time to recurrence considering the imbalances in the distributions of baseline characteristics. *P* values of the log-likelihood ratio test were obtained from the modeling. Interactions between polymorphisms and gender, race and type of adjuvant therapy on time to recurrence were tested by comparing corresponding likelihood ratio statistics between the baseline and nested Cox proportional hazards model that included the multiplicative product terms [9].

An internal validation analysis using bootstrapping was carried out to reduce the possibility of overfitting or biased conclusions [10]. One thousand bootstrap samples were generated from the original sample. Each bootstrap sample consisted of 125 observations drawn from the original dataset using simple random sampling with replacement [11]. Variables chosen in the original analysis retained in multivariable analysis if associated $P < 0.05$ in >50% of sample simulations.

All statistical tests were two sided. Analyses were carried out using the SAS statistical package version 9.1 (SAS Institute Inc., Cary, NC).

results

A total of 125 patients with stage III CRC were included in this analysis: 50 women (40%) and 75 men (60%) with a median age of 58 years (range 31–87 years). There were 70 Caucasian (56%), 22 Hispanic (18%), 26 Asian (21%) and seven African-American (6%) study participants. All patients were diagnosed with stage III CRC during the years of 1992 and 2007. The median follow-up was 4.2 years.

Fifty-nine of 125 patients had tumor recurrence, with a probability of 3-year recurrence of 0.45 ± 0.047. The median time to recurrence was 5.2 years (95% CI 2.5–11.1 years). Fifty-one of 59 (86%) patients showed recurrent disease within the first 3 years after surgery. Twenty-one patients showed one site of recurrence (36%), 22 patients (37%) displayed two sites of recurrence and 16 patients (27%) had three or more sites of recurrence. Thirty of 59 patients (51%) recurred in the liver, 46% (27 of 59) recurred in the lung, 47% (28 of 59) showed peritoneal carcinomatosis and 36% (21 of 59) recurred in other organs. Thirty-six of 125 patients have died and the median overall survival for the cohort is 11.9 years (95% CI 5.8 to 14.3+). Patients with fewer than 12 lymph nodes removed were more likely to develop tumor recurrence (median TTR of 2.5 years; CI 1.4 to 6.6), compared with patients with >12 lymph nodes removed (median TTR 7.1 years; CI 2.8 to 10.4+) (log-rank test $P = 0.045$). We did not observe any significant associations between other demographic and clinicopathologic variables and TTR. Detailed clinicopathologic characteristics are shown in Table 1.

Polymorphisms of *VEGF* and *IL-8* were not associated with demographic (age, gender and ethnicity), clinical (type of chemotherapy) or pathologic characteristics [tumor grade and N-stage (N1/N2); data not shown].

Table 1. Demographic and clinicopathologic characteristics and time to tumor recurrence in patients with stage III CRC

	n	Median TTR years (95% CI)	Relative risk (95% CI)	Probability ± SE[a] of 3-year recurrence	*P* value[b]
Age, years					0.69
≤50	29 (23.2%)	6.8[c] (2.3, 6.8+)	1 (reference)	0.46 ± 0.10	
>50	96 (76.8%)	5.2 (2.4, 11.1)	1.14 (0.60, 2.15)	0.45 ± 0.05	
Sex					0.63
Male	75 (60.0%)	5.2 (2.0, 11.1)	1 (reference)	0.45 ± 0.06	
Female	50 (40.0%)	5.7 (2.4, 10.4+)	0.88 (0.52, 1.49)	0.45 ± 0.07	
Race					0.12
White	70 (56.0%)	3.4 (1.8, 11.1)	1 (reference)	0.47 ± 0.06	
African-American	7 (5.6%)	2.3 (0.5, 3.3+)	2.04 (0.79, 5.24)	0.79 ± 0.18	
Asian	26 (20.8%)	7.1 (1.5, 7.7+)	0.83 (0.42, 1.64)	0.43 ± 0.10	
Hispanic	22 (17.6%)	10.4[c] (3.9, 10.4+)	0.52 (0.22, 1.23)	0.28 ± 0.11	
T stage					0.24
T1[d]	2 (1.6%)				
T2[d]	13 (10.4%)	7.4[c] (7.4+ ???)	1 (reference)	0.23 ± 0.12	
T3	93 (74.4%)	3.9 (2.3, 11.1)	3.02 (0.94, 9.72)	0.47 ± 0.06	
T4	13 (10.4%)	2.0 (1.0, 10.7+)	3.55 (0.92, 13.71)	0.57 ± 0.14	
Tx	4 (3.2%)	2.7 (1.3, 11.3+)	3.10 (0.61, 15.66)	0.50 ± 0.25	
N stage					0.52
N1	70 (56.0%)	6.6 (2.5, 11.3+)	1 (reference)	0.42 ± 0.06	
N2	55 (44.0%)	5.2 (1.7, 12.4+)	1.18 (0.71, 1.98)	0.48 ± 0.07	
No. of resected lymph nodes					0.045
<12	39 (31.2%)	2.5 (1.4, 6.6)	1 (reference)	0.56 ± 0.08	
≥12	86 (68.8%)	7.1 (2.8, 10.4+)	0.60 (0.36, 1.01)	0.40 ± 0.06	
Adjuvant therapy					0.69
5-FU	76 (60.8%)	3.9 (1.7, 12.4+)	1 (reference)	0.47 ± 0.06	
5-FU/LV/oxaliplatin	31 (24.8%)	3.4 (1.8, 4.2+)	0.99 (0.50, 1.94)	0.49 ± 0.12	
5-FU/LV/CPT-11	18 (14.4%)	7.1[c] (2.0, 7.1+)	0.71 (0.32, 1.58)	0.37 ± 0.12	
Tumor site					0.90
Left	69 (56.1%)	5.7 (1.8, 10.7+)	1 (reference)	0.44 ± 0.06	
Right[d]	53 (43.1%)	3.9 (2.0, 12.4+)	0.97 (0.57, 1.63)	0.46 ± 0.07	
Left and right[d]	1 (0.8%)				
Differentiation					0.34
Well[d]	4 (3.5%)				
Moderate[d]	70 (62.0%)	6.6 (2.6, 12.4+)	1 (reference)	0.41 ± 0.06	
Moderate/poor	39 (34.5%)	2.5 (1.7, 11.1+)	1.31 (0.75, 2.28)	0.51 ± 0.09	

[a]Greenwood SE.

[b]Based on log-rank test.

[c]Estimates were not reached.

[d]Grouped together for the estimates of relative risk and probability ± SE of 3-year recurrence.

CRC, colorectal cancer; TTR, time to recurrence; CI, confidence interval; SE, standard error; 5-FU, 5-fluorouracil; CPT-11, Irinotecan; LV, leucovorin.

VEGF C+936T and TTR in stage III disease

Sixty-six percent (80 of 121) of patients were homozygous for *VEGF* +936 C allele, 31% (37 of 121) were heterozygous (C/T) and 3% (four of 121) were homozygous for the 936 T allele. The *VEGF* C+936T polymorphism showed significant association with TTR. Patients with the *VEGF* +936 C/C homozygous genotype had a median TTR of 2.6 years (95% CI 1.7 to 5.7 years), compared with 11.1 years (95% CI 6.6 to 12.4+ years) in patients heterozygous or homozygous for the T allele ($P = 0.003$, log-rank test, Figure 1A).

IL-8 T−251A and TTR in stage III disease

Thirty percent (36 of 121) of patients were homozygous for the *IL-8* −251 T-allele, 51% (62 of 121) were heterozygous (T/A) and 19% (23 of 121) were homozygous for the −251 A allele. The *IL-8* T−251A polymorphism showed a significant association with TTR. Patients with the *IL-8* −251 A/A homozygous genotype had a median time to recurrence of 2.4 years (95% CI 1.0–3.9 years), compared with 6.6 years (95% CI 2.7 to 12.4+ years) for those with heterozygous −251 T/A allele and 5.7 years for homozygous −251 T-allele carriers (95% CI 1.8 to 11.3+ years) ($P = 0.048$, log-rank test, Figure 1B).

multivariable analysis of *IL-8* T−251A and *VEGF* C+936T

When we analyzed *IL-8* T−251A (adjusted *P* value = 0.030) and *VEGF* C+936T (adjusted *P* value<0.001) jointly, stratified by race, number of resected lymph nodes and type of adjuvant

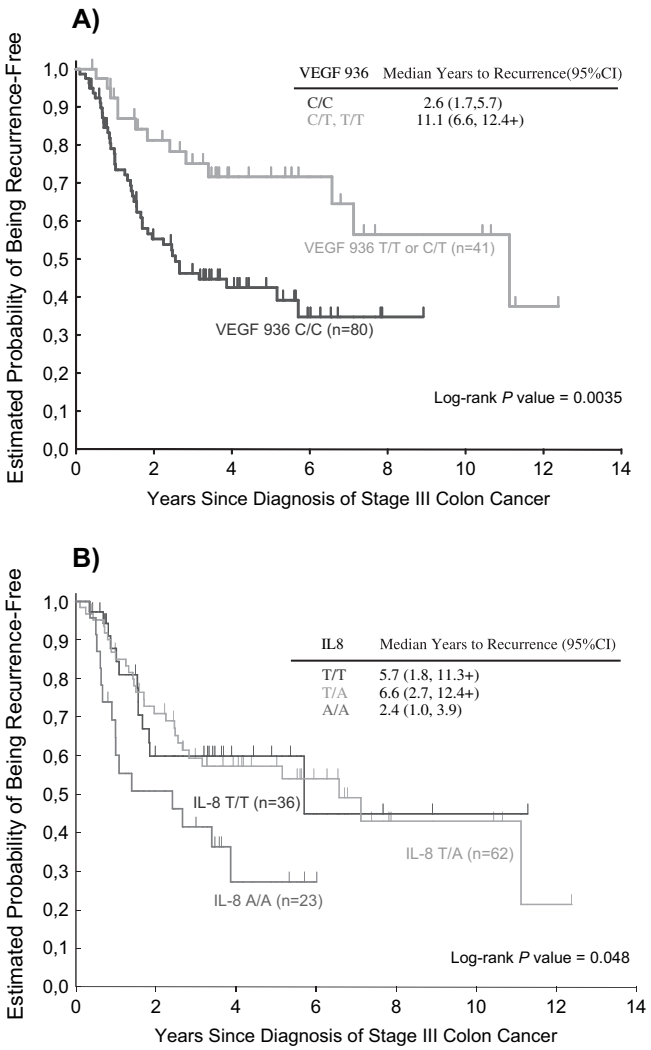

Figure 1. Recurrence-free survival of patients with stage III CRC by *VEGF* (A) and *IL-8* (B) polymorphisms.

therapy, the two polymorphisms remained significantly associated with time to recurrence (Table 4). Bootstrap analysis confirmed that polymorphisms were selected for the final multivariable model in 88% for *VEGF* C+936T and 56% for *IL-8* of the 1000 bootstrap samples as predictive factors significantly associated with time to recurrence at the 0.05 level.

In a combined analysis, there was a statistically significant relationship between the two polymorphisms and TTR. Patients with *VEGF* +936 C/C and *IL-8* −251 A/A genotype were at greatest risk to develop tumor recurrence (TTR = 1.0 year, CI 0.7–3.9), compared with patients displaying the combination of *VEGF* +936 T/T and *IL-8* −251 T/T genotype, who were less likely to develop tumor recurrence (TTR = 11.1 years, CI 7.1 to 12.4+) ($P < 0.001$, log-rank test; Figure 2).

analysis of interactions between *IL-8* T−251A and *VEGF* C+936T and sex, race and type of adjuvant therapy on time to recurrence

We tested whether the associations between *IL-8* T−251A and *VEGF* C+936T and time to recurrence differed by sex, race and type of adjuvant therapy. No significant interactions were found (data not shown).

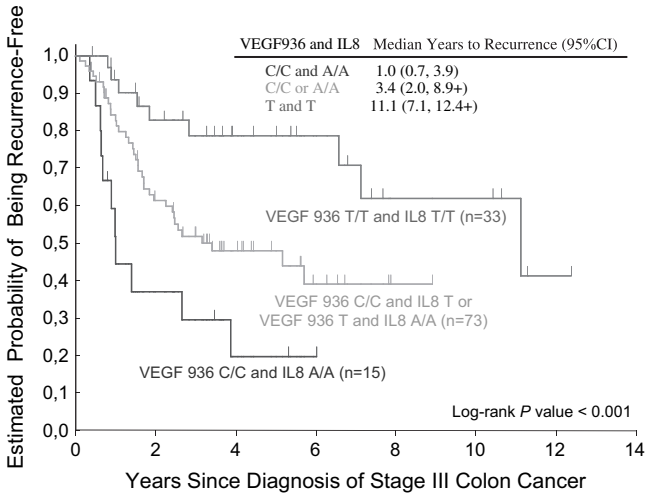

Figure 2. Recurrence-free survival of patients with stage III CRC by combination of *VEGF* and *IL-8* polymorphisms. Vertical hash marks, time of last follow-up for those patients who were still recurrence-free at the time of the analysis of data. All censored patients and those who were recurrent are accounted for.

analysis of other tested germline polymorphisms involved in the tumor angiogenesis pathway

We did not observe statistically significant associations between other tested genes involved in tumor angiogenesis pathway ($n = 12$) and TTR (Table 3).

discussion

We were able to demonstrate that germline polymorphisms of genes involved in the tumor angiogenesis pathway independently predict tumor recurrence in stage III CRC patients. To the best of our knowledge, this is the first study to show that angiogenesis-related germline polymorphisms may be important prognostic markers for CRC tumor relapse.

Vascular endothelial growth factor is one of the most important activators of tumor-associated angiogenesis [12]. Activation of the VEGF/VEGF-receptor axis triggers multiple signaling pathways that result in endothelial cell survival, mitogenesis, migration, differentiation, vascular permeability and mobilization of endothelial progenitor cells [13]. Overexpression of *VEGF* mRNA and protein has been associated with tumor progression and poor prognosis in a variety of malignancies, including melanoma, ovarian carcinoma, prostate carcinoma and colon carcinoma [14–16]. Its expression level in cancer cells directly correlates with tumor size, metastasis and poor prognosis in many types of solid and hematological tumors [13]. DNA sequence variations within the *VEGF* gene lead to altered VEGF production and/or activity. Several polymorphisms within the *VEGF* gene have been described. A C to T change at position +936 within the 3′-untranslated region of the *VEGF* gene has been associated with decreased plasma levels of VEGF, as shown by Renner

and coworkers [17]. Numerous studies reported on associations between *VEGF* C+936T polymorphism and susceptibility to cancer and other diseases, including breast and lung cancer [18–21] A recent study demonstrated that the prevalence of the *VEGF* +936T allele, which is associated with decreased plasma levels of VEGF, was less common in breast cancer patients than in healthy subjects, indicating that this genetic variant may be protective against breast cancer [18]. In addition, *VEGF* gene polymorphisms were found to be an independent prognostic marker for Korean patients with surgically resected gastric cancer [22]. To date, angiogenesis-related germline polymorphisms have not been

Table 2. Polymorphisms of genes in angiogenesis and time to recurrence in patients with stage III CRC

	n	Median time to recurrence (TTR) years (95% CI)	Relative risk (95% CI)	Probability ± SE[a] of 3-year recurrence	*P* value[b]
VEGF C+936T					0.003
C/C	80 (66.1%)	2.6 (1.7, 5.7)	1 (reference)	0.54 ± 0.06	
C/T[c]	37 (30.6%)	11.1 (6.6, 12.4+)	0.42 (0.22, 0.79)	0.25 ± 0.07	
T/T[c]	4 (3.3%)				
VEGF C-634G					0.76
G/G	43 (35.5%)	11.1 (1.7, 12.4+)	1 (reference)	0.41 ± 0.08	
G/C	60 (49.6%)	3.4 (2.0, 11.3+)	1.23 (0.69, 2.21)	0.48 ± 0.07	
C/C	18 (14.9%)	5.7 (1.5, 8.9+)	1.06 (0.46, 2.42)	0.38 ± 0.12	
VEGFR-2 $(AC)_n$ repeat					
11/9[c]	1 (0.8%)				
11/10[c]	1 (0.8%)				
11/11[c]	59 (48.8%)	7.1 (2.6, 12.4+)	1 (reference)	0.38 ± 0.07	0.17
11/12	50 (41.3%)	2.8 (1.8, 11.1+)	1.65 (0.95, 2.86)	0.50 ± 0.08	
12/12	10 (8.3%)	6.6 (0.9, 10.4+)	1.71 (0.65, 4.50)	0.49 ± 0.18	
Nrp1 3′-UTR C/T					0.26
C/C	35 (28.9%)	7.1 (2.8, 10.4+)	1 (reference)	0.35 ± 0.08	
C/T	51 (42.2%)	2.5 (1.7, 6.6)	1.56 (0.84, 2.88)	0.54 ± 0.07	
T/T	35 (28.9%)	11.1[d] (1.8, 11.1+)	1.03 (0.48, 2.17)	0.38 ± 0.09	
IL-8 T−251A					0.048
T/T	36 (29.8%)	5.7 (1.8, 11.3+)	1 (reference)	0.40 ± 0.09	
T/A	62 (51.2%)	6.6 (2.7, 12.4+)	1.06 (0.55, 2.06)	0.41 ± 0.07	
A/A	23 (19%)	2.4 (1.0, 3.9)	2.14 (1.01, 4.53)	0.58 ± 0.11	
CXCR1 G+2607C					0.83
G/G	95 (79.8%)	5.2 (2.5, 11.3+)	1 (reference)	0.43 ± 0.05	
G/C[c]	21 (17.7%)	11.1 (2.4, 12.4+)	0.93 (0.48, 1.82)	0.45 ± 0.11	
C/C[c]	3 (2.5%)				
CXCR2 C+785T					0.53
C/C	41 (35.3%)	2.7 (1.6, 11.1+)	1 (reference)	0.50 ± 0.09	
C/T	45 (38.8%)	7.1 (2.4, 10.7+)	0.70 (0.37, 1.32)	0.38 ± 0.08	
T/T	30 (25.9%)	3.2 (1.5, 12.4+)	0.83 (0.42, 1.62)	0.45 ± 0.09	
EGF A+61G					0.17
A/A	30 (24.8%)	5.7 (3.2, 12.4+)	1 (reference)	0.29 ± 0.09	
A/G	68 (56.2%)	6.6 (2.6, 11.3+)	1.10 (0.57, 2.12)	0.44 ± 0.06	
G/G	23 (19%)	1.5 (0.9, 6.8+)	1.91 (0.88, 4.16)	0.62 ± 0.11	
EGFR G+497A					0.53
G/G	50 (41.3%)	3.4 (2.3, 11.3+)	1 (reference)	0.47 ± 0.07	
G/A	60 (49.6%)	11.1 (2.4, 12.4+)	0.74 (0.42, 1.30)	0.43 ± 0.07	
A/A	11 (9.1%)	5.2 (1.7, 7.1+)	0.98 (0.42, 2.26)	0.36 ± 0.15	
EGFR $(CA)_n$ repeat					0.09
Both $(CA)_n < 20$	43 (38.7%)	2.4 (1.7, 6.6)	1 (reference)	0.55 ± 0.08	
Any $(CA)_n \geq 20$	68 (61.3%)	7.1 (3.2, 12.4+)	0.63 (0.36, 1.09)	0.37 ± 0.06	

[a]Greenwood SE.

[b]Based on log-rank test.

[c]Grouped together for the estimates of relative risk and probability ± SE of 3-year recurrence.

[d]Estimates were not reached.

CRC, colorectal cancer; TTR, time to recurrence; CI, confidence interval; SE, standard error; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; IL-8; interleukin-8; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor.

Table 3. Primer sequences, annealing temperatures and restriction enzymes

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Enzyme	Annealing
VEGF C+936T	AAGGAAGAGGAGACTCTGCGCAGAGC	TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG	*Nla*III	60°
VEGF C-634G	ACTTCCCCAAATCACTGTGG	GTCACTCACTTTGCCCCTGT	Seq.	60°
VEGFR-2 $(AC)_n$ repeat	GCTTGTAGTAATTGTTCATAAGTGG	GAGCGTATGTCTACT ATACGCCA	n.a.	60°
Nrp1 3′UTR C/T	AGCTTTGGTTGGTTTTGGTG	CCTGGAAACAAAAGGCATTC	Seq.	60°
IL-8 T−251A	TTGTTCTAACACCTGCCACTCT	GGCAAACCTGAGTCTCACA	*Mfe*I	60°
CXCR1 G+2607C	CTCATGAGGACCCAGGTGAT	GGTTGAGGCAGCTATGGAGA	*Alu*I	60°
CXCR2 C+785T	CATCTTTGCTGTCGTCCTCA	CTGTGAAGGATGCCCAGAAT	Seq.	60°
EGF A+61G	CATTTGCAAACAGAGGCTCA	TGTGACAGAGCAAGGCAAAG	*Alu*I	60°
EGFR G+497A	TGCTGTGACCCACTCTGTCT	CCAGAAGGTTGCACTTGTCC	*Bst-N*I	59°
EGFR $(CA)_n$ repeat	ACCCCAGGGCTCTATGGGAA	TGAGGGCACAAGAAGCCCCT	n.a.	55°

VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; seq., direct DNA sequencing; n.a., not available.

reported to be causatively linked to TTR or clinical outcome in adjuvant CRC patients. In our study, high-expression variants of *VEGF* C+936T (*VEGF* +936 C/C) were found to be significantly associated with TTR in both univariate and multivariable analysis (Table 2, Table 4, Figure 1A). These findings demonstrate for the first time that *VEGF* C+936T may be an important prognostic factor for stage III CRC, indicating a potential role of tumor-associated angiogenesis in the development of CRC tumor relapse.

Recently, IL-8, a member of the CXC chemokine family, has been found to be a critical VEGF-independent mediator of tumor-associated angiogenesis [23]. IL-8 exerts its potent angiogenic properties on endothelial cells through interaction with its receptors CXCR1 and CXCR2 [24, 25]. Induction of IL-8 preserved the angiogenic phenotype in HIF1-α-deficient colon cancer cells, suggesting a critical role of IL-8 in tumor-associated angiogenesis, independently of VEGF [23, 26]. Overexpression of *IL-8* has been found to be associated with VEGF-independent angiogenesis, advanced disease stage, lymphovascular invasion, poor prognosis and tumor recurrence in several different malignancies, including non-small-cell lung cancer and rectal cancer [23, 27, 28]. Additionally, CRC patients with lung and liver metastases have been found to have elevated plasma levels of IL-8 [29], indicating a potential role in VEGF-independent angiogenesis and tumor metastases. Hull et al. [30] identified a common single-nucleotide polymorphism −251 bp upstream of the *IL-8* transcription start site. In their *in vivo* models, they reported that the *IL-8* −251A allele was associated with increased plasma levels of IL-8 and further functional studies including promoter assays confirmed the functional role of the *IL-8* T−251A polymorphism [31, 32]. Recently, the *IL-8* −251 A-allele was reported to be associated with an increased risk of developing breast, prostate, gastric and CRC [31, 33–35]. As previously demonstrated by our group, *IL-8* T−251A and its receptor *CXCR1* were associated with clinical outcome in CRC patients [8, 27]. High-expression variants of *IL-8* T−251A were found to be significantly associated with risk of recurrence in rectal cancer patients in both univariate and regression tree analyses [27]. The present study shows that stage III CRC patients harboring high-expression variants of *IL-8* T−251A polymorphism (A/A genotype) were at higher risk of developing tumor recurrence supporting our hypothesis that increased angiogenic potential is critical for tumor relapse. A combined analysis of *VEGF* C+936T and *IL-8* T−251A showed a statistically significant relationship between the two polymorphisms and TTR. Grouping alleles into favorable versus nonfavorable alleles, high-expression variants of *VEGF* C+936T and *IL-8* T−251A (*VEGF* +936 C and *IL-8* −251 A) were associated with a higher likelihood of developing tumor recurrence (Table 4, Figure 2) ($P < 0.001$, log-rank test). In addition, multivariate analysis confirmed that VEGF C+936T (adjusted P value < 0.001) and *IL-8* T−251A (adjusted P value = 0.030) were significantly associated with TTR (Table 4).

Table 4. Multivariable analysis of VEGF and IL-8 polymorphisms and time to recurrence

	n	Adjusted RR (95% CI)[a]	Adjusted *P* value
VEGF C+936T			
C/C (unfavorable)	80 (66.1%)	1 (reference)	<0.001
C/T, T/T (favorable)	41 (33.9%)	0.28 (0.12, 0.64)	
IL-8 T−251A			
T/T, T/A (favorable)	98 (81%)	1 (reference)	0.030
A/A (unfavorable)	23 (19%)	2.24 (1.12, 4.47)	
Combined[b]			
2 favorable	33 (27.3%)	1 (reference)	<0.001
1 favorable	73 (60.3%)	5.04 (1.69, 15.0)	
0 favorable	15 (12.4%)	9.45 (2.74, 32.6)	

[a]Based on Cox proportional hazards model, stratified by race, number of resected lymph nodes and type of adjuvant therapy, with two polymorphisms included.
[b]Based on Cox proportional hazards model, stratified by race, number of resected lymph nodes and type of adjuvant therapy.
VEGF, vascular endothelial growth factor; IL-8; interleukin-8; CI, confidence interval; RR, response rate.

As with all retrospective, pilot studies, this analysis has potential limitations; nonetheless, this type of retrospective

study is an ideal forum for testing a novel hypothesis and generating data that can be confirmed in a prospective study. First, our findings are on the basis of a relatively small number of patients who were recruited over a period of 15 years; secondly, due to the retrospective setting of this study, traditional analyses of tumor angiogenesis such as microvessel density or venous/lymphatic vascular invasion were not possible and thirdly, we examined eight genes within the angiogenesis pathway. While it is recognized that the observed associations and patterns require confirmation with an independent dataset, and no amount of reanalysis with the current dataset will eliminate that need, we have taken care to (i) select the candidate genes with a documented role in the angiogenesis pathway, which have been found to be associated with prognosis in previous studies at our institution and/or in published manuscripts and (ii) perform an internal validation analysis to reduce the likelihood of overanalyzing this dataset. Therefore, the results of this pilot study should be interpreted carefully within the context of other publications and analyses.

Notwithstanding the aforementioned limitations, we have identified two independent molecular markers for tumor recurrence in stage III CRC. Consistent with our hypothesis, high-expression variants of *VEGF* C+936T and *IL-8* T−251A polymorphisms were independently associated with decreased TTR in stage III CRC patients, indicating a potential role of tumor angiogenesis in the development of tumor recurrence. Recently, targeted agents such as bevacizumab, an IgG1 mAb to the VEGF, have demonstrated relevant clinical activity in metastatic CRC [36]. Thus, the assessment of the patients' individual angiogenic potential on the basis of *VEGF* and *IL-8* genotypes might further enhance antiangiogenic treatment not only by the identification of patients who are at high risk but also by selecting more efficient treatment strategies. However, larger, independent and prospective biomarker-embedded clinical trials are needed to confirm and validate our preliminary findings.

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