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Potentially functional genetic variants in *KDR* gene as prognostic markers in patients with resected colorectal cancer

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Angiogenesis plays a key role in the development and treatment response of various tumors. The signaling transductions mediated by the binding of vascular endothelial growth factor (VEGF) to its receptor KDR (kinase insert domain receptor) is the most important pathway in tumor angiogenesis. Single nucleotide polymorphisms (SNPs) in *VEGF* have been extensively implicated in the etiology and treatment outcome of colorectal cancer (CRC). However, no study has been reported evaluating the role of *KDR* SNPs in CRC prognosis. We herein assessed the association between four potentially functional *KDR* SNPs and tumor recurrence in a Chinese population with 408 surgically resected CRC patients. The most significant SNP was for rs10013228 located in the *KDR* gene promoter. Compared with the homozygous wild-type genotype, the variant-containing genotypes of this SNP were significantly associated with a reduced recurrence risk with a hazard ratio (HR) of 0.53 (95% confidence interval [CI] 0.30–0.95, *P* = 0.032). Moreover, a borderline significant association was noted for another promoter SNP, rs2071559, with an HR of 0.67 (95% CI 0.42–1.07, *P* = 0.092). In stratified analysis, the associations of both SNPs were more prominent in patients receiving chemotherapy (HR = 0.47, 95% CI 0.23–0.94, *P* = 0.033 for rs10013228 and HR = 0.55, 95% CI 0.32–0.95, *P* = 0.032 for rs2071559). Further analysis revealed a protective effect on patient recurrence by chemotherapy (HR = 0.56, 95% CI 0.32–1.01, *P* = 0.046), which was more evident in patients with the variant-containing genotypes of each of the two SNPs (HR = 0.09, 95% CI 0.02–0.55, *P* = 0.009 for rs10013228 and HR = 0.39, 95% CI 0.18–0.86, *P* = 0.020 for rs2071559). Collectively, our findings suggest SNPs in the *KDR* gene modulate CRC recurrence, especially in those receiving chemotherapy. (*Cancer Sci* 2012; 103: 561–568)

Colorectal cancer (CRC) is the third most common malignancy and the fourth leading cause of cancer death worldwide.[1] CRC is generally recognized as a disease largely influenced by demographic characteristics and environmental exposures.[2] However, numerous recent studies have suggested that the risk of developing CRC is also modulated by inter-individual genetic variations.[3–5] Moreover, mounting evidence, including several of our previous studies, has demonstrated that single nucleotide polymorphisms (SNPs) are potential surrogate biomarkers for the genetic background of patients that can be used to predict the therapeutic response and prognosis of CRC patients.[6–9]

Angiogenesis is a process of new blood vessel formation from pre-existing vascular networks by capillary sprouting. Angiogenesis plays an important role in the growth, metastasis, and treatment response of many solid tumors including CRC.[10–12] Vascular endothelial growth factors (VEGFs) and their endothelial tyrosine kinase receptors are central regulators of angiogenesis.[13] The human VEGF family consists of five major ligands including VEGF, VEGFB, VEGFC, VEGFD, and placental growth factor (PIGF). These ligands bind to three different types of VEGF receptors (VEGFRs), including KDR (kinase insert domain receptor, also named VEGFR, VEGFR2, or FLK1), FLT4 (VEGFR3), and FLT1 (VEGFR1). Kinase insert domain receptor is the most important VEGF receptor that mediates the signaling transduction initiated by the VEGF ligands.[14,15] Vascular endothelial growth factor signaling through KDR is the main pathway that activates angiogenesis by inducing proliferation, survival, sprouting, and migration of endothelial cells, as well as increasing endothelial permeability. The cumulative effects of all these cellular processes mediated by the VEGF-KDR signaling cascade facilitates tumor growth, invasion, and therapeutic resistance.[16]

Most genetic association studies on the angiogenesis pathway in CRC have been focused on the SNPs of the *VEGF* genes. Although the expression alteration of KDR in CRC have been extensively reported,[17] to date, only a few studies have been published regarding *KDR* SNPs and CRC clinical outcomes with mixed results. A recent pilot study reported that two *KDR* SNPs were correlated with microvessel density and overall survival in a small population of 110 Denmark CRC patients.[18] In another phase II trial evaluating the efficacy of the combined treatment of chemotherapy plus bevacizumab, an anti-VEGF monoclonal antibody in metastatic CRC patients, Loupakis *et al.* analyzed five *VEGF* SNPs and three *KDR* SNPs and found there was no significant association between these polymorphisms and CRC outcomes.[19] Given the pivotal role played by KDR in the signaling transduction of the VEGF-dependent angiogenesis pathway, additional studies on *KDR* SNPs in CRC prognosis are warranted. In the present study, we sought to evaluate the effect of four potentially functional polymorphisms in the *KDR* gene on CRC recurrence in a Chinese population with 408 surgically resected CRC patients.

Materials and Methods

Study population. The population used in this study has been described previously.[7] Briefly, newly diagnosed and histologically confirmed CRC patients were enrolled in the Xijing Hospital and Tangdu Hospital affiliated with the Fourth Military Medical University in Xi'an, China. Patients with a history of

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other cancers were excluded from this study. The patient enrollment started from February 2006. The rate of recruitment among all eligible patients is 90%. As of April 2010, a total of 496 eligible CRC patients were recruited. For this study, 88 patients were excluded, including 26 patients who did not undergo surgery or only received palliative operation, 48 patients who had incomplete clinical and/or follow-up data, six patients who died within 1 month after surgery, and eight patients who had poor quality and/or quantity of DNA samples. Finally, a total of 408 patients with complete and validated demographic, clinical, and follow-up data were included in this study. All patients received surgery within 2 months after diagnosis and the primary tumor for all patients was completely resected, which was confirmed by pathological review of the tumors after resection. No patients received neoadjuvant chemotherapy or radiation therapy. Informed consent was obtained from the patients. This study was approved by the local research ethics committees of participating institutes.

Epidemiologic and clinical data collection. Demographic data were collected through in-person interviews at the time of initial visit or follow-up in the clinics, medical chart review, or consultation with the treating physicians by trained clinical research specialists. For data acquired from multiple sources, the research staff compared and validated the consistency of these data. The patients and/or family members were further contacted for verification if there were discrepancies. An individual who smoked more than 100 cigarettes in history was defined as an ever smoker, otherwise as a never smoker. Never drinkers were defined as those who never took alcohol or took less than or equal to one drink per month. One drink was defined as one bottle or can of beer, one medium glass of wine, or one mixed drink. Detailed clinical information was collected through medical chart review and/or consultation with the treating physicians. Tumor stage was assessed by American Joint Committee on Cancer (AJCC) staging system, and the lymph node invasive and organ metastasis information were included in different tumor stages. All patients with tumor stage 3 had lymph node invasion. All patients with tumor stage 4 had distant metastasis to other organs. Among the 39 patients at stage 4, 27 had liver metastasis, four had celiac metastasis, and eight had metastasis to other organs such as ovary, oviduct, vagina and lung. The follow-up information on patient recurrence and survival was updated at 6-month intervals through onsite interview, direct calling, or medical chart review. The latest follow-ups in this study were carried out in February 2011. Genomic DNA was obtained for each participant as described previously.[7]

SNP selection and genotyping. Potentially functional SNPs in the *KDR* gene were selected through the combined use of the datasets from the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/). Briefly, all SNPs in the potentially functional regions including exons, 5′ UTRs, 3′ UTRs, promoters (10 000 bp 5′ flanking region), and splicing sites were identified from the dbSNP database. We did not include any region further downstream of 3′ UTR because this region is generally not considered as a potentially functional region. In addition, using the Tagging SNP selection tool implemented in the HapMap, we identified all tagging SNPs of the *KDR* genes based on a linkage disequilibrium co-efficient $r^2 < 0.8$ and a minor allele frequency (MAF) < 0.05 in the Chinese population (CHB). The location of the tagging SNPs on the *KDR* gene was confirmed using dbSNP. From these SNPs identified from the dbSNP and HapMap database, we then filtered out SNPs that were non-validated, located in introns of the *KDR* gene, and/or had a minor allele frequency of <5% in Chinese. Finally, four SNPs remained after filtering and were genotyped in this study, including rs10013228 and rs2071559 in the promoter region, rs2305948 in exon 7, and rs1870377 in exon11. Genotyping was done using the Sequenom iPLEX platform (Sequenom Inc., San Diego, CA, USA). Laboratory personnel conducting genotyping were blinded to patient information. Strict quality control measures were implemented during genotyping with more than 99% concordance in samples that were randomly selected to be genotyped in duplicate.

Statistical and data analysis. The endpoint focused in this study is patient recurrence. Recurrence is defined as, after the resection of the primary tumor by surgery, the re-growth of tumor in the original organ (local-regional recurrence) or a different organ (distant metastasis).[20] All recurrent tumors in this study were confirmed as having the same histopathological characteristics as the primary tumor. Recurrence was confirmed through the combined evaluations of imaging findings (ultrasound, computed tomography, positron emission tomography, magnetic resonance imaging) and laboratory results (mainly the carcinoembryonic antigen test). Second primary tumors with different histopathological characteristics were excluded. The time to recurrence was defined as the time from initial surgery treatment to local recurrence, distant metastasis, or to the date of last follow-up. All patients without recurrence or lost to follow-up were censored for the analysis. Hazard ratios (HR) were estimated by multivariate Cox proportional hazards model, adjusting for age, gender, body mass index (BMI), education status, smoking status, drinking status, tumor position, tumor differentiation, tumor stage, and chemotherapy, where appropriate. Kaplan–Meier curve and log-rank test were used to assess the differences in time to recurrence between patients with different genotypes. SAS software package (version 9.2; SAS Institute, Cary, NC, USA) was used for the abovementioned analyses. All *P*-values in this study were two-sided. $P \leq 0.05$ was considered as the threshold of statistical significance.

Results

Characteristics of the study subjects. The distribution of patients' demographic and clinicopathologic characteristics is summarized in Table 1. A total of 408 CRC patients were included in this study. The average age at the time of CRC diagnosis was 59.4 (range, 22–90), and average BMI was 22.7 (range, 15.8–32.9). All patients received surgery within 2 months after CRC diagnosis. All patients had adenocarcinoma confirmed by histopathological examination. There were 230 (56.4%) male patients and 178 (46.6%) female patients. The majority of patients were never smokers (70.8%) and never drinkers (89.5%). There were approximately equal numbers of patients with colon cancer (47.1%) and rectal cancer (52.9%), which is consistent with previous reports that the incidence rates of colon and rectal cancers are generally of the same magnitude in countries with low CRC risk such as China.[21] A total of 192 (47.1%) patients had stage 2 tumor, while stage 0, 1, 3, and 4 tumors were presented in 2.0%, 14.2%, 27.2%, and 9.6% of patients, respectively. The majority of patients (66.2%) had moderately differentiated tumors. No patients received radiotherapy or targeted therapy, but most patients (78.2%) received adjuvant chemotherapy after surgery. Among the 319 patients receiving chemotherapy, 308 (96.6%) received the FOLFOX regimen, including folinic acid (FOL), fluorouracil (F) and Oxaliplatin (OX), as the first line treatment. During a median follow-up time of 23.7 months, there were 93 (22.8%) patients who developed recurrence. We further analyzed the association between important clinical variables and CRC recurrence risk in both univariate and multivariate Cox proportional hazards model analyses (Table S1). As expected, tumor stage was a significant predictor of patient recurrence in both univariate and multivariate analysis. Overall, chemotherapy was associated with a borderline significant reduced risk of recurrence in multivariate analysis (HR = 0.60, 95% 95% confidence interval [CI]

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Table 1. Demographic and clinicopathologic characteristics of the 408 colorectal cancer (CRC) patients

Variables	Number of patients (%), n = 408
Age, average (range) (in years)	59.4 (22–90)
Body mass index, mean (range)	22.7 (15.8–32.9)
Gender	
Male	230 (56.4)
Female	178 (43.6)
Smoking status	
Ever	119 (29.2)
Never	289 (70.8)
Drinking status	
Ever	43 (10.5)
Never	365 (89.5)
Education	
Up to high school	178 (43.6)
College degree or higher	171 (41.9)
Unknown	59 (14.5)
Tumor position	
Colon	192 (47.1)
Rectum	216 (52.9)
Tumor stage	
0	8 (2.0)
1	58 (14.2)
2	192 (47.0)
3	111 (27.2)
4	39 (9.6)
Organ of distant metastasis for stage 4 patients	
Liver	27 (69.2)
Other	12 (30.8)
Tumor differentiation	
Poor	37 (9.1)
Moderate	271 (66.4)
Well	100 (24.5)
Chemotherapy	
Yes	319 (78.2)
No	89 (21.8)
Recurrence	
Yes	93 (22.8)
No	315 (77.2)

0.34–1.07, $P = 0.084$). Tumor position and differentiation did not significantly influence CRC recurrence (Table S1).

Main effects of the four *KDR* SNPs on CRC recurrence. All patients in this study had adenocarcinoma and received surgical resection. Approximately 96.6% of chemotherapy-treated patients received the FOLFOX regimen. Therefore, in order to further enhance the homogeneity of this study and eliminate the confounding effect from chemotherapy treatment, we excluded those 11 patients who received non-FOLFOX chemotherapy agents. Finally, we had 397 patients who either did not receive chemotherapy or received FOLFOX-based chemotherapy for the downstream analyses. No strong linkage between these four SNPs was noted (Table S2). The average call rate of the four SNPs in this study was 99.2% (range, 98.0–99.8%). The detailed genotyping results are listed in Table 2. Because of the small number of patients with the homozygous variant genotypes in three of the four SNPs, we only tested the dominant genetic model (variant-containing genotypes versus homozygous wild-type genotype) to obtain optimum statistical power. Only one of the four SNPs, rs10013228 that is located in the promoter region of *KDR* gene, exhibited a statistically significant association with CRC recurrence. Compared with the homozygous wild-type (WW) genotype, the variant-containing (WV + VV) genotypes were associated with a significantly reduced CRC risk

Table 2. Associations of *KDR* single nucleotide polymorphisms (SNPs) with colorectal cancer (CRC) recurrence

SNP location nucleotide change	Genotype	Recurrence/ total	HR (95% CI)†	*P*-value
rs10013228	WW	68/277	1.00 (reference)	
Promoter	WV	19/102	0.62 (0.35–1.12)	0.112
−5578A>G	VV	0/12	NA	
	WV + VV		**0.53 (0.30-0.95)**	**0.032**
rs2071559	WW	40/165	1.00 (reference)	
Promoter	WV	35/170	0.74 (0.44–1.25)	0.260
−604T>C	VV	11/59	0.53 (0.26–1.08)	0.080
	WV + VV		0.67 (0.42–1.07)	0.092
rs2305948	WW	73/312	1.00 (reference)	
Exon 7	WV	12/78	0.60 (0.31–1.14)	0.120
1192G>A	VV	2/6	1.32 (0.30–5.76)	0.715
	WV + VV		0.65 (0.35–1.19)	0.163
rs1870377	WW	29/138	1.00 (reference)	
Exon 11	WV	38/156	1.04 (0.59–1.84)	0.884
1719A>T	VV	20/101	0.86 (0.45–1.65)	0.645
	WV + VV		0.98 (0.58–1.64)	0.926

The significant *P*-values (≤0.05) are in bold. †Adjusted for age, gender, education level, BMI, smoking status, drinking status, chemotherapy, tumor position, tumor differentiation, tumor stage. CI, confidence interval; HR, hazard ratio; VV, homozygous variant genotype; WW, homozygous wild-type genotype; WV, heterozygous genotype.

of recurrence with an HR of 0.53 (95% CI, 0.30–0.95, $P = 0.032$). Additionally, rs2071559, a SNP also located in *KDR* promoter, exhibited a borderline significant association with a reduced recurrence risk with an HR of 0.67 (95% CI, 0.42–1.07, $P = 0.092$). The two non-synonymous SNPs located in the exon regions did not show significant associations.

Stratified analyses of rs10013228 and rs2071559 by chemotherapy. Postoperative chemotherapy usually affects clinical outcome of CRC patients. Because the FOLFOX-based chemotherapy was the only first-line therapy in these patients after surgery, we further stratified the associations of *KDR* SNPs and CRC recurrence by chemotherapy to evaluate whether the significant KDR SNPs are prognostic markers for overall CRC or for specific groups of CRC patients receiving the FOLFOX chemotherapy. As shown in Table 3, the reduced risk of recurrence associated with both SNPs remained significant in those patients receiving chemotherapy but not in those without chemotherapy. The HR conferred by the variant-containing genotypes of rs10013228 was 0.47 (95% CI 0.23–0.94, $P = 0.033$) in patients receiving chemotherapy and 1.41 (95% CI 0.42–4.70, $P = 0.581$) in patients without chemotherapy. For rs2071559, the HR was 0.55 (95% CI 0.32–0.95, $P = 0.032$) and 1.08 (95% CI 0.22–5.39, $P = 0.922$), respectively. For the rs2305948 SNP that did not exhibit a significant association with CRC recurrence in the main effect analysis, the variant-containing genotypes were associated with a significantly reduced risk in those patients without chemotherapy (HR = 0.11, 95% CI, 0.01–0.95, $P = 0.045$) but not in those receiving chemotherapy (HR = 0.65, 95% CI 0.32–1.39, $P = 0.214$). Consistent with the Cox regression analysis, Kaplan–Meier curve indicated a borderline significant difference, in term of time to recurrence, between the wild-type and variant-containing genotypes of rs10013228 in all patients (Fig. 1A, left panel, log rank $P = 0.074$). This difference was more evident in patients with chemotherapy (Fig. 1A, middle panel, log rank $P = 0.057$), compared with those without chemotherapy (Fig. 1A, right panel, log rank $P = 0.860$). For rs2071559, no significant difference was observed between the homozygous wild-type and variant-containing genotypes on time to recurrence (Fig. 1B, left

Table 3. Associations of *KDR* single nucleotide polymorphisms (SNPs) with colorectal cancer (CRC) recurrence stratified by chemotherapy

SNP	Genotype	In patients with chemotherapy			In patients without chemotherapy		
		Recurrence/total	HR (95% CI)†	*P*-value	Recurrence/total	HR (95% CI)†	*P*-value
rs10013228	WW	55/218	1.00 (reference)	**0.033**	13/59	1.00 (reference)	0.581
	WV + VV	14/85	**0.47 (0.23–0.94)**		5/29	1.41 (0.42–4.70)	
rs2071559	WW	36/135	1.00 (reference)	**0.032**	4/30	1.00 (reference)	0.922
	WV + VV	33/171	**0.55 (0.32–0.95)**		13/58	1.08 (0.22–5.39)	
rs2305948	WW	58/243	1.00 (reference)	0.214	15/69	1.00 (reference)	**0.045**
	WV + VV	11/64	0.65 (0.32–1.29)		3/20	**0.11 (0.01–0.95)**	
rs1870377	WW	19/101	1.00 (reference)	0.443	10/37	1.00 (reference)	0.138
	WV + VV	50/206	1.29 (0.67–2.47)		8/51	0.33 (0.08–1.42)	

The significant *P*-values (≤0.05) are in bold. †Adjusted for age, gender, education level, BMI, smoking status, drinking status, tumor position, tumor differentiation, tumor stage. CI, confidence interval; HR, hazard ratio; VV, homozygous variant genotype; WW, homozygous wild-type genotype; WV heterozygous genotype.

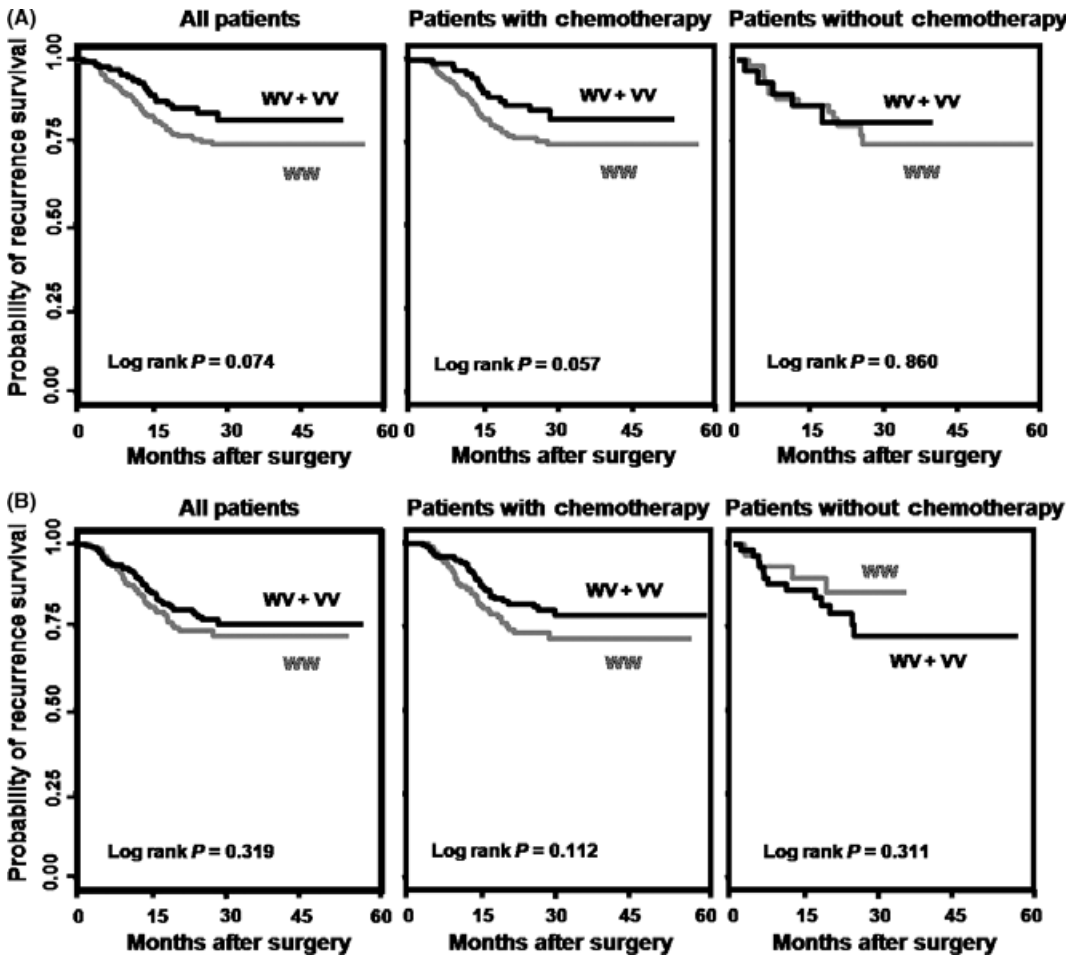

Fig. 1. Kaplan–Meier curves and log rank tests for (A) rs10013228 and (B) rs207155.

panel, log rank $P = 0.319$). However, in chemotherapy-stratified analysis, the difference became more significant in patients receiving chemotherapy (Fig. 1B, middle panel, log rank $P = 0.112$) compared with those without chemotherapy (Fig. 1B, right panel, log rank $P = 0.311$). Taken together, the data from both the Cox regression and the log rank analyses suggested that the reduced risks of CRC recurrence associated with both rs10013228 and rs2071559 were more prominent in patients with chemotherapy.

The effects of chemotherapy on CRC recurrence modulated by *KDR* SNPs. Since adjuvant chemotherapy is generally given to CRC patients to prevent recurrence after the primary tumors are surgically removed, we further evaluated the association between the FOLFOX chemotherapy with CRC recurrence as well as whether such an effect was modulated by the significant SNPs identified in this study. As shown in Table 4, patients receiving the FOLFOX chemotherapy exhibited a significant lower recurrence risk compared to those without chemotherapy

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Table 4. Effects of chemotherapy on colorectal cancer (CRC) recurrence modulated by *KDR* single nucleotide polymorphisms (SNPs)

Genotype and variables	Recurrence/total	HR (95% CI)†	*P*-value
In all patients			
No chemotherapy	18/89	1.00 (reference)	**0.046**
Chemotherapy	69/308	**0.56 (0.32–1.01)**	
In patients with WW genotype of rs10013228			
No chemotherapy	13/59	1.00 (reference)	0.283
Chemotherapy	55/218	0.69 (0.35–1.36)	
In patients with WV + VV genotype of rs10013228			
No chemotherapy	5/29	1.00 (reference)	**0.009**
Chemotherapy	14/85	**0.09 (0.02–0.55)**	
In patients with WW genotype of rs2071559			
No chemotherapy	4/30	1.00 (reference)	0.845
Chemotherapy	36/135	1.12 (0.35–3.60)	
In patients with WV + VV genotype of rs2071559			
No chemotherapy	13/58	1.00 (reference)	**0.020**
Chemotherapy	33/171	**0.39 (0.18–0.86)**	

The significant *P*-values (≤0.05) are in bold. †Adjusted for age, gender, education level, BMI, smoking status, drinking status, tumor position, tumor differentiation, tumor stage. CI, confidence interval; HR, hazard ratio; VV, homozygous variant genotype; WW, homozygous wild-type genotype; WV heterozygous genotype.

(HR = 0.56, 95% CI 0.32–1.01, $P = 0.046$). This favorable prognosis conferred by chemotherapy was more prominent in patients with variant-containing genotypes (low-recurrence risk genotype) of both rs10013228 and rs2071559 (HR = 0.09, 95% CI 0.02–0.55, $P = 0.009$; and HR = 0.39, 95% CI 0.18–0.86, $P = 0.020$, respectively). In comparison, no significant effect conferred by chemotherapy on CRC recurrence was observed in patients with the high-recurrence risk genotypes of both SNPs ($P = 0.283$ for rs10013228 and $P = 0.845$ for rs2071559).

Combined effects of the protective alleles of rs10013228 and rs2071559. To test whether there is a cumulative effect of the *KDR* SNPs on CRC prognosis, we performed a combined analysis of the protective alleles (the allele conferring a reduced risk of recurrence) in rs10013228 and rs2071559. Using subjects without the protective allele as the reference group, those carrying greater than or equal to three protective alleles exhibited a significant reduced risk of CRC recurrence in overall patients (HR = 0.23, 95% CI, 0.07–0.77, $P = 0.017$) and in patients with chemotherapy (HR = 0.20, 95% CI, 0.05–0.82, $P = 0.026$) (Table 5). These associations were in a dose-dependent manner, as evidenced by a significant result in a trend test ($P_{trend} = 0.017$ for overall patients and $P_{trend} = 0.010$ for patients receiving chemotherapy). In comparison, the combined effects of the protective alleles were not observed in patients without chemotherapy (HR = 0.74, 95% CI, 0.04–15.33, $P = 0.845$, $P_{trend} = 0.951$) (Table 5).

Joint effects KDR SNPs and previously identified significant risk loci for CRC recurrence. We further analyzed the joint and interaction effects between two significant KDR SNPs and rs4779584 or rs10795668, two SNPs that were previously found to be associated with CRC recurrence in this patient cohort.[7] As shown in Table S3, a significant joint effect was observed between rs10795668 and either rs10013228 or rs2071559. For both combinations, compared with patients with the wild-type of both SNPs, those with the variant-containing genotypes for both SNPs had a significantly reduced recurrence risk with an HR of 0.30 ($P < 0.01$ for both). However, no significant interaction was noted for these analyses (Table S3).

Discussion

In the current study, we evaluated the association between four potentially functional polymorphisms in the *KDR* gene and recurrence risk in a Chinese population with 408 CRC patients. Two SNPs, rs10013228 and rs2071559, both in the promoter region of *KDR*, were identified to confer at least a borderline significant association with a reduced risk of CRC recurrence. Additional analyses revealed that the combined alleles of the two SNPs conferred a more prominent effect on CRC recurrence, especially in patients treated by the FOLFOX chemotherapy agents.

The rationale of this study to investigate the role of pivotal angiogenesis genes in CRC clinical outcome is based on the hypothesis that tumor progression and treatment response are angiogenesis-dependent and can be influenced by anti-angiogenic therapy. This hypothesis has been confirmed experimentally by a large body of evidence over the past several decades, which originated in 1971 from the publication by Folkman *et al.*[22] It has been generally recognized that the most important tumor angiogenesis signaling transduction is mediated by the interaction between VEGF and its receptor, KDR, a protein expressed in most endothelial cells. The binding of VEGF to KDR leads to the activation of a wide plethora of downstream signaling pathways, such as the upregulation of the PLCγ-PKC-MEK-MAPK pathway that mediates cell proliferation, and the activation of the PI3K-AKT pathway that promotes endothelial cell survival.[12,16] Various independent studies have reported an elevated expression of KDR in CRC development. For example, Takahashi *et al.*[23] reported that the increased expression of both VEGF and KDR significantly correlated with the vascularity, progression, and metastasis of CRC. Moreover, it has also been demonstrated that KDR influences the treatment response and prognosis of CRC patients.[24–26] For instance, Calvani[26] recently found that the inhibition of KDR protein function abrogated VEGF-mediated activation of HIF1A, which in turn increased the survival of the HCT116 colon cancer cells that are sensitive to bevacizumab, the most commonly used

Table 5. Combined analysis of protective alleles of *KDR* single nucleotide polymorphisms (SNPs) with colorectal cancer (CRC) recurrence

No. protective alleles†	Overall			In patients with chemotherapy			In patients without chemotherapy		
	Recurrence/total	HR (95% CI)‡	*P*-value	Recurrence/total	HR (95% CI)‡	*P*-value	Recurrence/total	HR (95% CI)‡	*P*-value
0	40/164	1.00 (reference)		36/134	1.00 (reference)		4/30	1.00 (reference)	
1–2	43/188	0.78 (0.50–1.29)	0.357	31/139	0.68 (0.38–1.21)	0.192	12/49	1.12. (0.22–5.74)	0.894
≥3	3/36	**0.23 (0.07–0.77)**	**0.017**	2/28	**0.20 (0.05–0.82)**	**0.026**	1/8	0.74 (0.04–15.33)	0.845
P for trend			**0.017**			**0.010**			0.951

The significant *P*-values (≤0.05) are in bold. †The protective alleles included the G allele of rs10013228 and the C allele of rs2071559. ‡Adjusted for age, gender, education level, BMI, smoking status, drinking status, chemotherapy, tumor position, tumor differentiation, tumor stage, where appropriate.

anti-angiogenesis therapy in CRC treatment. Taken together, these lines of evidence highlighted the essential role of KDR in the etiology and clinical outcome of CRC, through mediating VEGF-induced signaling transductions.

Several studies have reported that genetic variations of the *KDR* gene were implicated in the risk and outcome of common human diseases, such as cardiovascular diseases,[27,28] chronic myeloid leukemia,[29] glioblastoma,[30] breast cancer[31] and CRC.[18,19,32,33] However, the roles played by individual *KDR* SNPs and their physiological functions in these studies remained unknown. In CRC, some studies were unable to identify a significant association between *KDR* SNPs (including rs2071559, rs2305948 and rs1870377) and patient outcome.[19,32,33] In comparison, one study reported an association between both rs2071559 and rs2305948 with microvessel density, as well as the progression-free survival of CRC patients receiving surgical treatment.[18] However, the latter study used a univariate, instead of a multivariate, approach to analyze the associations between *KDR* SNPs and CRC outcome, which might be biased by various confounders commonly associated with CRC prognosis.[18] In the current study, we identified an association between the rs10013228 SNP and a reduced recurrence risk. This finding from the Cox proportional hazards model was consistent with the results obtained from the Kaplan–Meier survival analyses and log rank tests, which indicated a significant difference between the patients with the homozygous wild-type genotype and the variant-containing genotypes in terms of the time to recurrence (Fig 1A, left panel). We also identified a borderline significant association for the rs2017559 SNP. These findings are biologically plausible since both SNPs are located in *KDR* promoter and have the potential to influence the gene expression of *KDR*. To date, no studies have been reported to evaluate the molecular and cellular functions of the rs10013228 SNP. We conducted a bioinformatics analysis of the region harboring rs10013228 using the UCSC Genome Browser (http://genome.ucsc.edu) to search functional elements and using the MultiZ and the PhyloP programs to compare the degree of conservation of the region across 46 vertebrates. Several highly conserved regions were identified at upstream of rs10013228, although no functional elements were found near this SNP (data not shown). Future *in vitro* studies are needed to determine whether rs10013228 is a causal variant or in linkage with other functional locus. A couple of independent studies have demonstrated the physiological significance of rs2071559 which is located in the −604 position of *KDR* gene promoter. Using luciferase reporter assay, Wang *et al.*[27] reported that the variant allele of this SNP conferred a reduced transcriptional activity, and was associated with a reduced KDR protein level, as well as a decreased risk of coronary heart disease. Consistently, Zhang *et al.*[28] reported an association between the variant allele of rs2071559 with a decreased carotid intima media thickness as well as a reduced risk of stroke and recurrence. In both studies, the reduced disease risk was suspected to be, at least partially, mediated by the decreased KDR expression level conferred by the variant allele of rs2071559. In accordance, our study found that the variant allele of this SNP was also associated with a reduced risk of recurrence of CRC patients. Meanwhile, the favorable prognostic effect conferred by chemotherapy was more prominent in patients with the variant-containing genotypes. Taken together, the evidence in both the current and previous studies suggested a direct causal role of rs2071559. Future molecular characterizations are needed to validate the functions of this SNP in CRC prognosis. Moreover, a comprehensive analysis based on a tagging SNP approach is necessary to evaluate the joint and interaction effects of all the SNPs in the *KDR* gene on CRC prognosis.

FOLFOX chemotherapy is commonly used as an adjuvant treatment for CRC patients after surgery. However, there are a wide spectrum of responses to this therapy in CRC patients, which are likely influenced by the genetic background and tumor status of the patients. It has been suggested that SNPs in pivotal drug action pathway genes may modulate the effects of treatments such as chemotherapy.[34] Therefore, we further conducted stratified analyses to determine whether the significant associations observed in the main effects analysis were modulated by chemotherapy. We found that the reduced risk of recurrence conferred by both rs10013228 and rs2071559 were more evident in patients receiving the FOLFOX chemotherapy than those without chemotherapy, which were evidenced by the lower HRs and more significant *P*-values in chemotherapy-treated patients. In addition, the patients without chemotherapy exhibited an increased HR for both SNPs, suggesting the presence of potential interactions between the genotypes of these two SNPs and chemotherapy in the modulation of CRC recurrence. However, a formal test of interaction was not significant for both SNPs (data not shown), possibly due to the inadequate power for interaction resulting from the modest sample size of the study. Moreover, we further demonstrated a protective effect conferred by the FOLFOX chemotherapy on patient recurrence, which was significantly stratified by both rs10013228 and rs2071559 (Table 4). The HR conferred by chemotherapy reduced from 0.56 to 0.09 and 0.39 when the analysis was restricted to patients with the variant-containing genotypes of rs10013228 and rs2071559, respectively (Table 4). Similar results were observed when we combined the low-risk alleles of the two SNPs in a joint analysis (Table 5). Collectively, these data suggested that the two SNPs might modulate the response of CRC patients to the FOLFOX chemotherapy individually and jointly. If validated, the *KDR* SNPs may be developed as potential biomarkers to select CRC patients to receive the FOLFOX treatment. In accordance with these findings, several reports have shown that angiogenesis factors are important in the response to the FOLFOX regimen and prognosis of CRC patients.[35,36] Furthermore, angiogenesis inhibitors targeting the VEGF-mediated pro-angiogenic pathway have exhibited clinically appreciable benefits for many types of cancers including CRC.[37] Moreover, several recent randomized controlled clinical trials have demonstrated a significant and clinically meaningful improvement in the survival of patients with metastatic CRC treated with the combined administration of anti-angiogenic target therapies and the FOLFOX chemotherapy.[38–40] It is worthwhile to further evaluate the prognostic and predictive roles of the *KDR* SNPs in the treatments that combine anti-angiogenic modalities and traditional chemotherapies.

A major strength of this study is the homogenous patient population. The CRC patients in this study were enrolled from Xi'an and adjacent areas in China. This region is attractive in conducting population-based research due to the geographical stability with low mobility rate. In addition, all the patients in this study had adenocarcinoma and were surgically treated to remove the primary tumor. Furthermore, the analyses were restricted to those patients receiving the first-line adjuvant FOLFOX chemotherapy. These highly homogenous patient characteristics and treatments substantially reduced the confounding effects of the heterogeneous therapeutics modalities observed in many other biomarker studies of cancer prognosis. The limitation of our study includes the generalizability issue. Since our study was restricted to Han Chinese, it remains a task for further evaluations whether the findings can be applied to other populations. In addition, the possibility of chance findings in our study could not be ruled out due to the relatively short follow-up time (median follow-up time, 23.7 months) and modest sample size. Nonetheless, the enrollment of this population is ongoing with a low rate of patient loss to follow-up, which will enable us to validate our findings and conduct more in-depth analyses in the future.

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In summary, we demonstrated a significant association of potentially functional genetic variations in the *KDR* gene with the risk of recurrence in CRC patients. Independent studies are warranted to validate these findings before their clinical applications.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The associations between selected clinical variables and colorectal cancer (CRC) recurrence risk.

Table S2. Linkage disequilibrium coefficient r^2 between the four single nucleotide polymorphisms (SNPs).

Table S3. Joint effects between the two single nucleotide polymorphisms (SNPs) and previous identified two SNPs.

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