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ORIGINAL ARTICLE

VEGF gene polymorphisms and susceptibility to colorectal cancer disease in Italian population

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Abstract
Background The vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen involved in the process of angiogenesis, a crucial phase in tumor growth and metastasis. We carried out a case–control study to evaluate whether polymorphisms of VEGF gene modulate the risk of developing colorectal cancer disease (CCD).
Materials and methods We evaluated VEGF −2578A/C, −460T/C, and +405C/G genotypes obtained from a series of 302 CCD patients and 115 controls from the Italian population using polymerase chain reaction restriction fragment length polymorphism assay.
Results Strong linkage disequilibrium (LD) was detected between −2578A/C and −460T/C (D'=0.97; CI=0.93–1) and between −2578A/C and +405C/G (D'=0.97; CI=0.98–1) in the case group. Complete LD was detected between −2578A/C and +405C/G and between −460T/C and +405C/G (D'=1; CI=0.84–1; CI=0.82–1, respectively) in the control group. A reduced risk for the disease was associated with −2578C/A and −2578C/C (odds ratio (OR)=0.34, CI=0.162–0.676 and OR=0.38, CI=0.181–0.775, respectively). A direct association was found for carriers of the VEGF −460C/C polymorphism (OR=3.55; CI=1.659–8.469). We identified a protective haplotype −2578A, −460T, and +405G (OR=0.04; CI=0.009–0.19) and two different high-risk haplotypes −2578A, −460C, and +405G (OR=1.90; CI=1.31–2.27) and −2578C, −460C, and +405C (OR=9.62; CI=1.3–70.87).
Conclusions The present study suggests that the VEGF gene polymorphisms may play a role in the development of colorectal cancer.

Keywords VEGF · Polymorphism · Colorectal · Cancer · SNP

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Introduction

The VEGF is an endothelial cell-specific mitogen involved in a number of pathologic processes, including angiogenesis, a crucial phase in the development of solid malignancies; tumor growth and metastasis are in fact angiogenesis dependent [1, 2].

Numerous studies have shown that growing tumors require the establishment of a blood supply [3], and VEGF is often up-regulated in cancer [4]. Indeed, interruption of the VEGF action profoundly inhibits tumorigenesis [5, 6].

Markers in the VEGF gene have been associated with increased risk of developing cancer, and recent studies have also demonstrated that the expression of the VEGF family had a prognostic significance in patients with cancer [7].

Polymorphisms in the VEGF gene not only affect the predisposition and the aggressiveness of cancer disease but are also associated with the pathogenesis of psoriasis [8], acute renal allograft rejection [9], sudden infant death syndrome [10], and predisposition to chronic kidney disease [11], and they may also contribute to an increased seriousness in ankylosing spondylitis [12] disease.

The VEGF gene is located on chromosome 6p12 and includes a 14-kb coding region with eight exons and seven introns [13], and it is polymorphic with at least 15 single nucleotide polymorphisms (SNPs) described [14–16]; some of these have been reported to be associated with differential expression of the VEGF in vitro and have been implicated as candidate markers in several diseases with a putative angiogenic basis [17].

Two of these SNPs −2578C/A and −460C/T (Genebank Accession Nos. RS699947 and RS833061, respectively) are located in the VEGF promoter region [14–16], and Shahbazi and colleagues found that peripheral blood mononuclear cells from −2578C/C homozygous individuals produced significantly more VEGF than cells from −2578 A/A homozygotes [9].

Another polymorphism +405C/G (Genebank Accession No. RS2010963) located in exon 1 of the VEGF gene [14] was observed to be significantly correlated with VEGF protein production, showing lowest VEGF protein production for C/C homozygotes and highest production for G/G homozygotes [14].

Stevens et al. showed that haplotypes containing the common polymorphisms at −460C in the promoter and +405G have a 71% higher basal promoter activity when compared with the wild-type sequence [18].

Based on the above considerations, our aim was to investigate whether the VEGF −2578C/A, −460C/T, and +405C/G gene polymorphisms are associated with an increased risk of developing CCD. We therefore analyzed the allele and haplotype frequencies of these polymorphisms and LD among them.

Haplotype association analyses and a case–control study with matched pairs among three VEGF polymorphisms were performed to understand their individual contribution to the disease.

Materials and methods

Genotyping

Blood samples were obtained from 302 individuals with CCD taken from three medical oncology units in Central Italy (Rome, Pesaro, and Livorno) and from a control group of 115 adult healthy donors with no family history of CCD.

CCD diagnosis was confirmed by independent anatomic pathologists after bioptical analysis. Eligibility criteria (Caucasian ethnicity and Italian residency) were verified during an interview with the designated investigators at each participating institution.

Family history was also traced back to at least three generations and laterally to second- and third-degree relatives to exclude hereditary nonpolyposis colorectal cancer. The mean age was 71 years for patients and 59 for controls.

The male/female ratio was 177:125 for cases and 54:61 for controls.

The genomic DNA was extracted from at least 200 μl of whole blood from each subject using the "salting out" method [19].

The VEGF genotypes were identified by restriction fragment length polymorphism polymerase chain reaction. Briefly, each PCR amplicon was digested by a specific restriction endonuclease. The digested DNA materials were then analyzed by 2% agarose gel electrophoresis, and genotypes were determined distinguishing between digested and undigested PCR products. The assays were performed as described in literature; primers' sequences, restriction enzymes, and references are shown in Table 1.

The study was approved by local ethics committees; patients and controls provided written informed consent.

Statistical analysis

The Hardy–Weinberg equilibrium of alleles at individual loci was tested to compare the observed genotype frequencies with the expected genotype frequencies among the subjects (http://www.genes.org.uk/software/hardy-weinberg.shtml).

McNemar's test was used to compare paired proportion in the single SNP-based association analysis; three genetic models (i.e., codominant, dominant, and recessive) were tested for individual SNP with matching for age and sex; ORs with 95% confidence intervals (CI) were used to measure the association of CCD with the genotype frequencies observed. Values of $p \leq 0.05$ were considered significant.

Lewontin's standardized disequilibrium coefficient (D') among all SNP markers was assessed using the Haploview program [20].

This software provides the disequilibrium coefficient D' as a measure of the nonrandom association of alleles at different loci. D' coefficient is equal to 1 only if two SNPs have not been separated by recombination (or recurrent mutation) during the history of the sample (complete LD).

According to the criteria of Gabriel et al., we define pairs to be in "strong LD" if the one-sided upper 95% confidence bound on D' is >0.98 (that is, consistent with no historical recombination), and the lower bound is above 0.7 [21].

Table 1 Characteristics of the studied polymorphisms with primer sequences and restriction enzymes

Primers	Restriction enzyme	Polymorphism	Reference
VEGF −2578			
F5′-GGC CTT AGG ACA CCA TAC C	*Bst*YI	−2578A/C	29
R5′-CAC AGC TTC TCC CCT ATC C			
VEGF −460			
F5′-TGT GCG TGT GGG GTT GAG ***C***G	*Bst*UI[a]	−460T/C	14
R5′-TAC GTG CGG ACA GGG CCT GA			
VEGF +405			
F5′-ATT TAT TTT TGC TTG CCA TT	*Bsm*FI	+405C/G	14
R5′-GTC TGT CTG TCT GTC CGT CA			

C base changed to create restriction enzyme site

[a] Restriction site created by base change in the forward primer

Haploview was also used to perform haplotype analyses, and the Chi-square test was used to compare the haplotype frequencies of cases and controls. Subjects who had missing data with at least one polymorphism were excluded from the haplotype analysis.

All significant associations were corrected for multiple testing, applying a Bonferroni correction by dividing the significance level by the number of major haplotypes for haplotype-based association analysis.

We used unconditional logistic regression and the most common haplotype in the control group (C–T–C) as the reference to estimate haplotype-specific ORs. Chi-square statistics and *p* values were also verified using MedCalc® (MedCalc, Mariakerke, Belgium).

All case data were used to perform LD and haplotype analysis; as a consequence of the 1:1 matched association performed to reduce age and sex biases and because of a younger control population, all case data from patients born before 1938 were censored in the single SNP-based association analysis.

Results

Three VEGF gene polymorphisms (−2578A/C, −460T/C, and +405C/G) were determined in 302 colorectal cancer diseased patients and 115 good health individuals.

The Hardy–Weinberg test in the study population confirmed that all genotypes were in equilibrium (data not shown).

Distribution of genotypes and the ORs estimated for association among the three genetic variants and CCD are shown in Table 2.

The results show that heterozygous carriers of the VEGF −460T/C polymorphism were not associated with CCD.

A significantly reduced risk for the disease was associated with −2578C/A and −2578C/C (OR=0.34, CI=0.162–0.676 and OR=0.38, CI=0.181–0.775, respectively) and with heterozygous carriers for the VEGF +405G polymorphism (OR=0.38; CI=0.197–0.714). A borderline inverse association was also found for the VEGF +405G/G polymorphism (OR=0.5; CI=0.250–0.959).

A direct association was instead found for homozygous carriers of the VEGF −460C polymorphism (OR=3.55; CI=1.659–8.469).

Strong LD was detected between −2578A/C and −460T/C (D'=0.97; CI=0.93–1) and between −2578A/C and +405C/G (D'=0.97; CI=0.98–1) but not between −460T/C and +405C/G (D'=0.72; CI=0.64–8) in the case group. Complete LD was detected between −2578A/C and +405C/G and between −460T/C and +405C/G (D'=1; CI=0.84–1; CI=0.82–1, respectively) but not between −2578A/C and −460T/C (D'=0.86; CI=0.74–0.94) in the control group.

We determined haplotype frequencies of three VEGF biallelic polymorphisms (−2578A/C, −460T/C, +405C/G), and of the eight possible haplotypes, only six were estimated to be present in the study population (Table 3). The C–T–C and C–C–G haplotype frequencies were similar in both case and control groups, and there was, therefore, no association between these haplotypes and CCD risk.

The A–C–G haplotype, the most common in the case group (43%), showed a significant increased risk of developing CCD (OR=1.8; CI=1.19–2.72); this direct association remained significant after Bonferroni correction (p=0.043).

A very strong direct association with the disease was seen to be related with the C–C–C haplotype (OR=10.78; CI=1.45–80.37). In contrast, the A–T–G haplotype showed a protective role against the CCD (OR=0.05; CI=0.012–0.25).

Discussion

Several studies now suggest a strong correlation between VEGF expression and both poor prognosis and metastasis in colorectal cancer. In particular, a large meta-analysis, including 27 studies [22], demonstrated that VEGF overexpression is significantly correlated with poor overall survival and with an increased risk of relapse.

A number of studies evaluating patient tumor tissue now propose that increased VEGF expression in primary colorectal cancer may predict the risk of multiple liver metastases [23, 24] and may play a role in the spread of

Table 2 Distribution of genotypes and ORs estimated for the association among three genetic variants and CCD

Genotype	Case	Control	OR	95% CI	*p* value
VEGF −2578					
AA	55 (18%)	12 (10%)	1.00	Reference	–
AC	150 (50%)	60 (52%)	0.343	0.162–0.676	0.0001
CC	97 (32%)	43 (37%)	0.387	0.181–0.775	0.0061
CA/CC	247 (82%)	103 (90%)	0.235	0.114–0.447	0.0013
VEGF −460					
TT	70 (23%)	47 (42%)	1.00	Reference	–
TC	153 (51%)	54 (49%)	0.976	0.615–1.546	1
CC	76 (25%)	10 (9%)	3.556	1.659–8.469	0.0006
TC/CC	229 (77%)	64 (58%)	0.978	0.630–1.516	1
VEGF +405					
CC	48 (16%)	15 (16%)	1.00	Reference	–
CG	135 (45%)	46 (51%)	0.385	0.197–0.714	0.0017
GG	118 (39%)	30 (33%)	0.5	0.250–0.959	0.0369
CG/GG	253 (84%)	76 (84%)	0.319	0.166–0.581	<0.001

The number of subjects of patients and controls may not reach the total due to genotyping failure
OR odds ratio adjusted for age and sex, *CI* confidence interval adjusted *p* value from McNemar's test

colorectal cancer cells to the lymph nodes [25]. Such evidence offered a strong rationale for the development of novel antiangiogenesis drugs interfering with the VEGF pathway in order to cut off the tumor's blood supply. A monoclonal antibody directed against VEGF, bevacizumab (Avastin®), has proven effective and was approved for the treatment of metastatic CCD.

Likewise, several studies were conducted to elucidate the role of VEGF common polymorphisms and VEGF protein production showing that haplotypes containing the common polymorphisms at −460C and +405G have a 71% higher basal promoter activity when compared with the wild-type sequence [18]; Koukourakis et al. reported a low VEGF expression linked with the −2578C/C genotype, while the −2578C/A was linked with high VEGF expression in nonsmall cell lung cancer [26].

Accordingly, with this evidence, we found that VEGF −2578C/C polymorphism was associated with a reduced risk of colorectal cancer, with an OR of 0.387 in the codominant model and an OR of 0.235 in the dominant model. Heterozygous carriers show an OR of 0.343 that is stronger than homozygous carriers, probably due to a wider heterozygous population that allows us to establish more confidently the risk for this category.

Similarly, the VEGF +405C/G confers significant protection against cancer (OR=0.385), but it is not so strong for homozygous carriers of the G allele, probably because of the low number of this category. In fact, the dominant model showed an increased protection value with an OR of 0.319. Moreover, VEGF +405G/G, due to the LD among SNPs, is over-represented in the most common haplotype in the case group.

On the contrary, VEGF −460 C/C showed an increased risk for disease with an OR of 3.556. No association was found for the heterozygous carriers or for the dominant model. This is probably due to the mechanism of coinheritance with homozygous −2578A and +405G showed by homozygous carriers of the −460 C/C mutation.

Confident that the theoretical expectation that haplotype-based analysis is more powerful than single-marker analysis

Table 3 VEGF haplotypes and ORs estimated for risk associations

Haplotypes			Frequencies			OR (95% CI)	*p* value	*p* value[a]
−2578	*−460*	*+405*	All	Case	Control			
C	T	C	0.341	0.328	0.390	Ref	–	–
A	C	G	0.397	0.429	0.281	1.79 (1.19–2.72)	0.0072	0.043
C	T	G	0.172	0.156	0.227	0.82 (0.517–1.317)	0.493	–
C	C	C	0.043	0.053	0.003	10.78 (1.446–80.373)	0.0081	0.048
C	C	G	0.026	0.027	0.022	1.39 (0.451–4.27)	0.756	–
A	T	G	0.017	0.003	0.070	0.05 (0.012–0.25)	<0.0001	<0.001

[a] Bonferroni-corrected *p*

[27, 28], we performed this kind of association that essentially could elucidate what haplotype could be associated with an increased or a diminished risk of developing CCD, but it could be also useful to clarify the meaning of the different distribution of the LD phenomenon between cases and controls and for a direct comparison with the single SNP-based association results.

The results of the haplotype analysis are shown in Table 3, and there were a total of six estimated haplotypes out of eight possible haplotypes in this study population.

We found two major haplotypes that are diversely represented between case and control groups: C–T–C (−2578, −460, +405) with a frequency of 39% in the control group and 32% in the case group and A–C–G with a frequency of 28% in the control group and 43% in the case group.

The most common haplotype in the case group, A–C–G, was seen to be associated with an increased risk of developing the disease (OR=1.79). Although C–C–C haplotype's frequency is very low in the whole population, the different distribution between cases and controls is clearly significant, making this haplotype a candidate risk factor for developing colorectal cancer (OR=10.78).

Instead, a strong inverse association with the disease was found with A–T–G haplotype (OR=0.05).

In conclusion, the present study is the first to provide evidence of the risk association of allelic variants of VEGF with CCD in the Italian population.

We identified a protective haplotype −2578A, −460T, and +405G and two different high-risk haplotypes −2578A, −460C, and +405G and −2578C, −460C, and +405C.

Considering the single SNP association analyses that highlights a risk role for the −460C homozygous variant and the different distribution of the LD of SNPs between case and control groups, we believe that VEGF −460C may be the only polymorphism, among the three studied SNPs, with a significant role in the etiology of CCD, but it must be taken into account that it is probably due to the existence of a LD between this SNP and another putative etiological variant (either within VEGF or a neighboring gene) not evaluated in this study.

If the −460T/C polymorphism were etiological and the C variant a dominant disease causing allele, all haplotypes containing the −460C allele would be expected to be positively associated with the disease and should therefore be over-represented in cases; this appears to be almost true (Table 3). On the contrary, if the −2578C allele were dominantly protective, then all C-containing haplotypes should be over-represented in controls (Table 3); it seems evident except for those haplotypes containing the −460C allele, but it could be explained thanks to the LD between these two SNPs.

These results suggest a possible role for the VEGF gene polymorphisms in the etiology of CCD.

Further studies evaluating the prognostic and predictive value to anti-VEGF targeted therapies in metastatic CCD of the allelic variants examined here are ongoing.

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Conflicts of interest statement The authors have declared no conflicts of interest.

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