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Prognostic Effect of Vascular Endothelial Growth Factor +936C/T Polymorphism on Tumor Growth Pattern and Survival in Patients Diagnosed with Colon Carcinoma

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Abstract

Introduction: Vascular endothelial growth factor (VEGF) is considered as endothelial cell-specific mitogen that plays an important role in the process of angiogenesis, thereby affecting the prognosis of tumor as angiogenesis is a crucial phase in tumor growth and metastasis. Accordingly, we carried out a case-control study to assess whether VEGF rs3025039 polymorphism affects the growth pattern and susceptibility to colon carcinoma.

Materials and methods: One hundred and fifty, formalin fixed paraffin embedded (FFPE) tissue samples from patients diagnosed with colon carcinoma and the same number of blood controls were used in the present study. VEGF +936 C>T (rs3025039) polymorphism was evaluated by pyrosequencing. Computer image analysis was used to analyse the growth pattern of the colon carcinoma tumor by using cytokeratin-8 stained slides.

Results: A heterozygous genotype TC in rs3025039 polymorphism was found as a significantly protective genotype as compared to homozygous genotypes (CC and TT). However we found no significant correlation between investigated polymorphisms, tumor growth pattern, 5 years survival and other clinicopathological parameters.

Conclusion: We concluded that the heterogenous genotype of VEGF rs3025039 polymorphism appears to be a protective factor for colon carcinoma that could be a useful marker in follow-up studies and may be a genetic determinant for colon carcinoma.

Keywords: Cell specific mitogen; Angiogenesis; Complexity index

Introduction

Colorectal cancer (CRC) has become one of the leading causes of cancer related deaths worldwide with more and more people being diagnosed for this disease every year. CRC is considered as the third most common type of cancer in developed countries, with a lifetime risk of 5%. About 1 million new cases of CRC are diagnosed with 600 000 deaths worldwide each year [1,2]. In Sweden, more than 6000 people are diagnosed with CRC annually. At the time of diagnosis, one out of five patients already has metastases (38) In advance cases; it is reported to metastasize to liver, peritonium and lymph nodes. So the factors which are involved in metastasis can be studied for their prognostic significance in tumors [3]. Angiogenesis, new blood vessels formation from endothelial precursors, is pre-requisite for tumor growth because of its intrinsic relation with metastasis [4]. During physiologic angiogenesis in adults, there is a temporary shift to pro-angiogenic factors, which is inhibited by anti-angiogenic factors and this mechanism remains balanced [5,6]. In pathological states, many proteins play important roles in blood vessel formation leading to tumor growth and metastasis. A good example is the vascular endothelial growth factor, VEGF. VEGF is an endothelial cell specific mitogen that is involved in various cellular and pathological processes including angiogenesis. Angiogenesis is a crucial phase in the tumor development; tumor growth and metastasis are in fact dependent upon agiogenesis which is well explained by many researchers [7-9]. VEGF is known to increase the vascular permeability of cells, as a result of this ability, it is also known as vascular permeability factor (VPE). Reports show that VEGF has potential to increase the vascular permeability of cells by more than 50,000 folds compared to histamine, which is believed as a standard for permeability [10]. It allows the diffusion of proteins to make a network into the interstitium which helps endothelial cells to migrate [10]. Tumors, that have developed

new blood vessels have capability to grow more rapidly along with high metastatic potential [10,11]. Since the expression of VEGF is very vital in the process of angiogenesis which can pave a way for metastasis of colorectal cancer, blocking angiogenesis by hindering the expression of VEGF can be used as an alternative treatment of colorectal cancer.

Amongst the many factors which are present in cells, VEGF has been extensively studied because of its specificity for the vascular endothelium [11-13] and found upregulated in many cancers [7,14,15]. Indeed, inhibiting the VEGF action results in the inhibition of tumorogenesis [16,17]. Several markers have been studied in this gene, which are associated with development of cancer and in a recent study, VEGF expression has been demonstrated as a prognostic marker in cancer patients [18].

There are more than 15 single nucleotide polymorphisims (SNPs), that are reported in different types of cancers [19,20]. Polymorphism in this gene has been associated with high risk of developing colorectal cancer and considered as an independent prognostic marker in this disease [21,22]. VEGF has been evaluated for different SNPs such as +936C>T (rs3025039), -2578C>A (rs699947), -1154G>A (rs1570360),

-634G>C (rs2010963), -460C>T (rs833061), and +405C>G (rs2010963) [23]. Among these polymorphisms, VEGF+936C>T (rs3025039) is one of the polymorphisms that has been significantly associated with different types of cancer such as oral and breast carcinoma [24,25]. For this reason, we wanted to see if there was an association between VEGF+936C>T (rs3025039) polymorphism and clinico-pathological parameters in colon can carcinoma patients compared to a healthy control group.

Tumor growth pattern and its size are important variables to consider during the evaluation of a tumor. Two types of growth patterns can be seen, infiltrative and expansive, among them, the latter has worse prognosis [26]. Considering tumor growth pattern as an important prognostic marker in the current study, a computer-based tumor growth pattern analysis technique called "complexity index was used to evaluate the invasiveness of tumor progression. The fractal dimension and the number of tumor cells and tumor cell clusters were used to estimate the grade of tumor complexity [27]. Complexity index value ranges between 1-5, in which 1 indicates a tumor with smooth and regular border while a tumor with complexity index value of 5 has highly irregular borders and even splits up into tumor cells/clusters [27].

Although many studies are available which show the association between VEGF polymorphism and susceptibility to colorectal cancer, very little is known about the effect of VEGF rs3025039 polymorphism on tumor growth pattern, 5 years survival as well as clinico-pathological parameters of the patients diagnosed with colon carcinoma. Since VEGF is significantly important in angiogenesis of CRC, it is reasonable to hypothesize that polymorphism in VEGF is a good candidate as a prognostic marker in development of colon carcinoma.

The aim of this study was to investigate the association between VEGF polymorphism and susceptibility to colon carcinoma. To our knowledge, this is the first study in which VEGF rs3025039 polymorphism is evaluated along with tumor growth pattern, complexity index, correlated with 5 years survival and clinico-pathological parameters of patients diagnosed with colon carcinoma.

Materials and Methods

Materials

A total of 150 FFPE tissue samples investigated in this study were randomly selected from the patients diagnosed for colorectal carcinoma at different tumor and age stages. All the patients were diagnosed between 2002-2009 and were collected from Örebro University Hospital, Örebro, Sweden. Blood from 150 healthy plasma and blood donors was used as controls. The study was approved by the ethical committee EPN, Uppsala, Sweden.

DNA extraction and primer designing

Tumor area was marked by an experienced morphologist (VH-S). Depending upon the size of the tumor area, 1-2 punches of 2 mm diameter were taken from the FFPE blocks. DNA from FFPE tissues was extracted by using Nucleospin® Nucleic acid and protein purification kit (Macherey-Nagel, Germany) according to manufacturer's protocol. DNA extraction kit (Macherey-Nagel, Germany) was used to extract DNA from blood and plasma according to manufacturer's instructions. Concentration and quality of DNA was analysed by NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

Primers were designed by using Pyro Mark Assay Design 2.0 software (Qiagen sample and Assay technology, Hilden, Germany) and

then subjected to PCR for optimization. Primer sequences (forward, reverse and sequencing primers) and their annealing temperatures are given in Table 1.

Polymerase chain reaction (PCR)

A master mix of KAPA2G buffer M (1X), reverse and forward primer (0.25 µM) (Biomers.net, GmbH, Germany), $MgCl_2$ (1 mM), deoxyribonucleotide triphosphate (dNTPs, 200 µM), KAPA2G Fast Hot Start polymerase (1U) (KAPA Biosystem, Boston Massachusetts, USA) and genomic DNA (90-100 ng) was prepared. PCR reactions were carried out in thermal cycler 2720 Gene Amp® (Applied Biosystems, Foster city, USA) in three steps. The first step included denaturation at 95°C for 10 min, followed by a second step of 49 PCR cycles with denaturation at 94°C for 45 s, annealing temperature (according to optimized annealing temperature of primers) for 30 s, and extension at 72°C for 30 s. Finally an extension was completed at 72°C for 7 min.

After amplification, PCR product was confirmed by gel electrophoresis. A 2% solution of agarose was prepared by adding High-resolution agarose (Sigma-Aldrich, Co. USA) to 1X TBE (Tris base, Acetic acid and EDTA) buffer solution. Band length of amplified product was compared by MassRuler™ DNA ladder low ranges (Ferments AB, Sweden) and visualized by ultraviolet trans-illuminator (Bio-Rad laboratories, AB, Sweden).

Pyrosequencing

Polymorphism in VEGF (rs3025039) was examined by PyroMark Q96 ID (Qiagen.Biotage AB, Uppsala, Sweden) according to manufactures protocol. In brief, A Streptavidin Sepharose™ Beads solution was prepared by adding MQ water and 1X binding buffer (1 mM/L EDTA, 0.1% Tween 20, 2 M/L NaCl, 10 mM/L Tris-HCl, Milli-Q water, pH 7.6) and added to 96 well PCR plate followed by the amplified PCR product from each sample. A sequencing primers solution was also prepared by adding 1X annealing buffer (2 mM/L Magnesium acetate, 20 mM/L Tris-Acetate, pH 7.6). PyroMark Q96 vacuum workstation (Qiagen, Germany) was used to purify the biotinylated PCR product Polymorphisms were analyzed by PyroMark ID 1.0 software (Biotage AB, Uppsala, Sweden). Substrate mixture, enzymes and dNTPs were added in the cartridge according to calculation by the software in Pyromark. Pyromark Q96 gold reagent kit (Qiagen, Hilden, Germany) was used according to manufacturer's instruction (Qiagen, Hilden, Germany).

Complexity index

To analyse tumor growth pattern, 40 samples from the same patients grouped for VEGF polymorphism study, were randomly selected. Slide preparations (sectioning and staining) and image processing was performed using the same method described by Franzen et al. [27]. Briefly, Slides with tumor sections were stained with cytokeratin 8 and images of tumor stromal area were captured by a camera mounted over the microscope at 10x. These images were processed to get tumor area black with white background in order to

Primer	Sequence	Annealing temperature (ºC)
Forward (F1)	AGACTCCGGCGGAAGCATT	60
Reverse (R1)*	CTTCCGGGCTCGGTGATT	
Sequence (S1)	CGGGCGGGTGACCCA	
*=Biotinylated		

Table 1: Primer sequences for VEGF rs3025039 polymorphism along with annealing temperatures.

measure the fractal dimensions and number of tumor cells/clusters in each slide. These two measurements, fractal dimension and number of cells were used to calculate complexity index.

Statistical analysis

SPSS, version 20 (SPSS Inc., Chicago, IL, USA) was used for statistical measurements. Continuous variables were measured as mean and standard deviations. Univariant binary logistic regression was applied to determine different SNPs as risk factor for colon cancer. To check trends, Pearson chi square test was used appropriately. Complexity index association was measured by Fisher's exact test. Using Kaplan–Meier's test, analyzed survival. P ≤ 0.05 was considered statistically significant.

Results

Patient characteristics

A total of 150 patients diagnosed with colon carcinoma were included in current analysis. The patient group was comprised of 68 (45.33%) males and 82 (54.67%) females, with a mean age 71 years (range, 47-95 years). Patients were divided into two age groups. Group 1 includes patients <70 years age while group 2 includes patients ≥70 years. Forty two (28%) patients were in group 1 while 108 (72%) were included in group 2. Tumor wall penetration stages T1, T2, T3 and T4 were 3 (2%), 14 (9.33%), 72 (48%) and 7 (4.67%), respectively. Lymph node metastasis N was categorized as N0, N1 and N2 with 59 (39.33%), 19 (12.67%) and 18 (12%) patients respectively. For tumor metastasis, 59 (63.33%) were at Mx stage while only one patient was diagnosed with as M1. In localization, 100 (66.67%) patients were diagnosed with right colon carcinoma and 5 (3.33%) with left side tumor localization. When analysing differentiation of colon carcinoma, 24 (16%) were low differentiated 94 (62.67%) medium and 27 (18%) highly differentiated. Patients were also categorized according to Duke's tumor staging system in which 17 (11.33%) of the patients were at stage A, 72 (48%) were at B, 51 (34%) at C and 5 (3.33%) were at stage D.

VEGF polymorphism and colon carcinoma

The distribution of VEGF rs3025039 polymorphism and its association with development of colon carcinoma was analysed (Table 2). A significant association was observed between TC genotype and colon carcinoma (p=0.027, OR=0.547, 95% CI=0.320-0.933). CT genotype seems to be a protective genotype in development of colon carcinoma. Frequencies for other two genotypes were CC 215 (71.67%) and TT 9 (3.0%) in our studied population (Figure 1).

VEGF polymorphism, tumor growth pattern and survival analysis

We have divided the complexity index variable into 3 groups, low complexity index (CI=1), medium complexity index (CI=2 and 3) and high complexity index (CI=4 and 5). There were 2 (5%) patients with low complexity index, 17 (42.5%) with medium complexity index, and in the high complexity index group, 21 (52.5%) patients. When we compare different genotypes of VEGF polymorphism with complexity index groups, there was no significant differences between growth pattern of tumor and VEGF rs3025039 polymorphism (p=0.482) (Table 3 and Figure 2).

The relationship between different genotypes of VEGF rs3025039 polymorphism and 5 -years survival of the patients was also evaluated. We could not find any significant association (p=0.705) between these two variables. From 114 (76.0%) patients with CC genotype, 51(34.0%) were alive and 63 (42.0%) died. In the TC genotype there were 29 (19.03%) patients from which 11 (7.3%) were alive and 18 (12%) died. Similarly, from 7 (4.7%) patients with TT genotype, 3 (2.0%) were alive and 4 (2.7%) died within 5 years after being diagnosed with colon carcinoma (Table 4).

Clinicopathological significance of VEGF polymorphism

In the analysis of VEGF rs3025039 polymorphism and clinicopathological parameters, there was no statistically significant association observed between VEGF polymorphism and any of the clinical parameters as can be seen in Table 5.

Discussion

In recent years, the search for prognostic biomarkers for colorectal cancer has been one of the most competitive areas in research. In cancer, one reason is the complex nature of tumor angiogenesis which is one of the known hallmarks of cancer. Angiogenesis is a sequence of different processes that starts with the dilation in pre-existing vessels followed by the proliferation of endothelial cells, formation of new blood vessels and recruitment of the perivascular cells. Therefore, angiogenesis is a key factor in tumor progression and metastasis. VEGF is one of the growth factors that are well known for controlling the angiogenesis process [28,29].
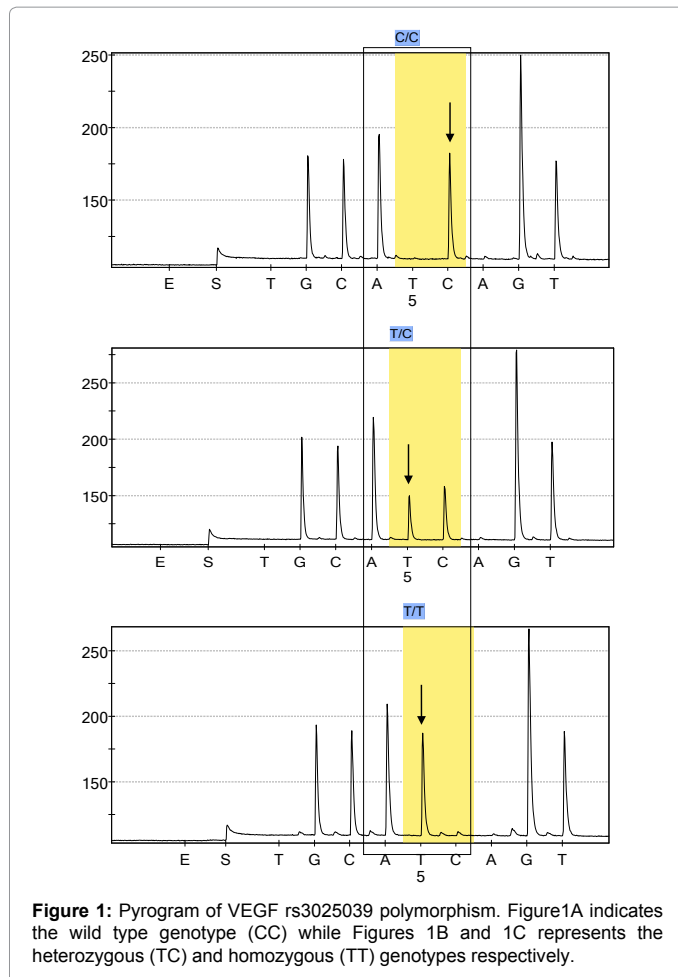
In this study, we have analyzed VEGF rs3025039 polymorphism in colon carcinoma patients and its relation with tumor growth pattern, 5 years survival of the patients and clinico-pathological parameters of patients diagnosed with colon carcinoma is studied. Since this polymorphism has been seen associated with other types of carcinoma and tumor progression in a higher relevance than other SNPs in VEGF. We randomly selected 150 patients diagnosed with colon carcinoma and the same number of healthy controls. VEGF rs3025039 polymorphism distribution in our population was as 76.0% CC genotype, 19.33% TC genotype and 4.67% TT genotype in patients while 67.33% CC, 31.33% TC and 1.33% were TT genotypes in the healthy control population.

Statistical analysis indicates that heterozygous genotype (TC) in this polymorphism appears to be a protective measure for colon carcinoma (p=0.027, OR=0.547, 95% CI=0.320-0.933) as compared to homozygous genotypes; CC and TT. This indicates that the C>T allelic change has more protective affect than a wild type genotype CC. This is a novel observation and was not previously reported. The association is quite unexpected as in previous studies, controversial roles of TC genotype in colorectal cancer has been described. According to the findings of Bae et al. [30] T bearing alleles at rs3025039 are responsible

Genotypes	Normal n (%age)	Tumors n (%age)	Total n (%age)	P value	OR	95% CI	
						Lower value	Upper value
CC	101 (67.33%)	114 (76.0%)	215 (71.67%)	Reference	1		
TC	47 (31.33%)	29 (19.33%)	76 (25.33%)	0.027	0.547	0.32	0.933
TT	2 (1.33%)	7 (4.67%)	9 (3.0%)	0.164	3.101	0.63	15.269
n=number, OR=Odds Ratio, CI=Confidence Interval							

Table 2: VEGF rs3025039 polymorphism and disease development in colon carcinoma.

Figure 1: Pyrogram of VEGF rs3025039 polymorphism. Figure1A indicates the wild type genotype (CC) while Figures 1B and 1C represents the heterozygous (TC) and homozygous (TT) genotypes respectively.

for colon cancer development. Similar results has been described by other investigators showing the C>T allele change as responsible for high risk of colorectal cancer development [31,32]. On the other hand, some researchers reported no significant difference between C or T allele frequency in patients and controls [33,34]. Similarly, Wu et al. [35] reported that VEGF +936 C/C genotype or C allele is not linked to CRC development. This inconsistency could be explained by, variation in the studied population, sample size as well as different sources of DNA and analysing techniques.

VEGF polymorphism was correlated with 5-years survival of the patients to see if any relationship between these two factors exists. We did not find any significant association (P>0.05) between 5 years survival and genetic variation in VEGF rs3025039 polymorphism. Previous reports show that +936 TT genotype is associated with worst survival in CRC patients as compared with CC genotype [21,33]. This contradiction could be due to the reason that our findings are based upon the low prevalence of TT genotype in our studied population.

Tumor growth pattern is well known for its prognostic significance and complexity index is a reliable method to measure growth pattern of tumor in CRC [27,36]. To examine any correlation between VEGF +936C>T polymorphism and tumor growth pattern, we calculated the complexity index and compared the results with different genotypes of VEGF polymorphism. Results indicate that there is no significant association between VEGF polymorphism and tumor growth pattern in patients diagnosed with colon carcinoma (p>0.05). As the aetiology

Genotype	Live n(%age)	Died n(%age)	Total n (%age)	P value
CC	51 (34.0%)	63 (42.0%)	114 (76.0%)	0.705
TC	11 (7.3%)	18 (12.0%)	29 (19.3%)	
TT	3 (2.0%)	4 (2.7%)	7 (4.7%)	
n=number				

Table 3: Genotypes of VEGF rs3025039 polymorphism and their association with 5 years survival of the patients diagnosed with colon carcinoma.

Figure 2: Kaplan Meier's survival curve showing association between different genotypes of VEGF rs3025039 and 5 years survival of patients diagnosed with colon carcinoma.
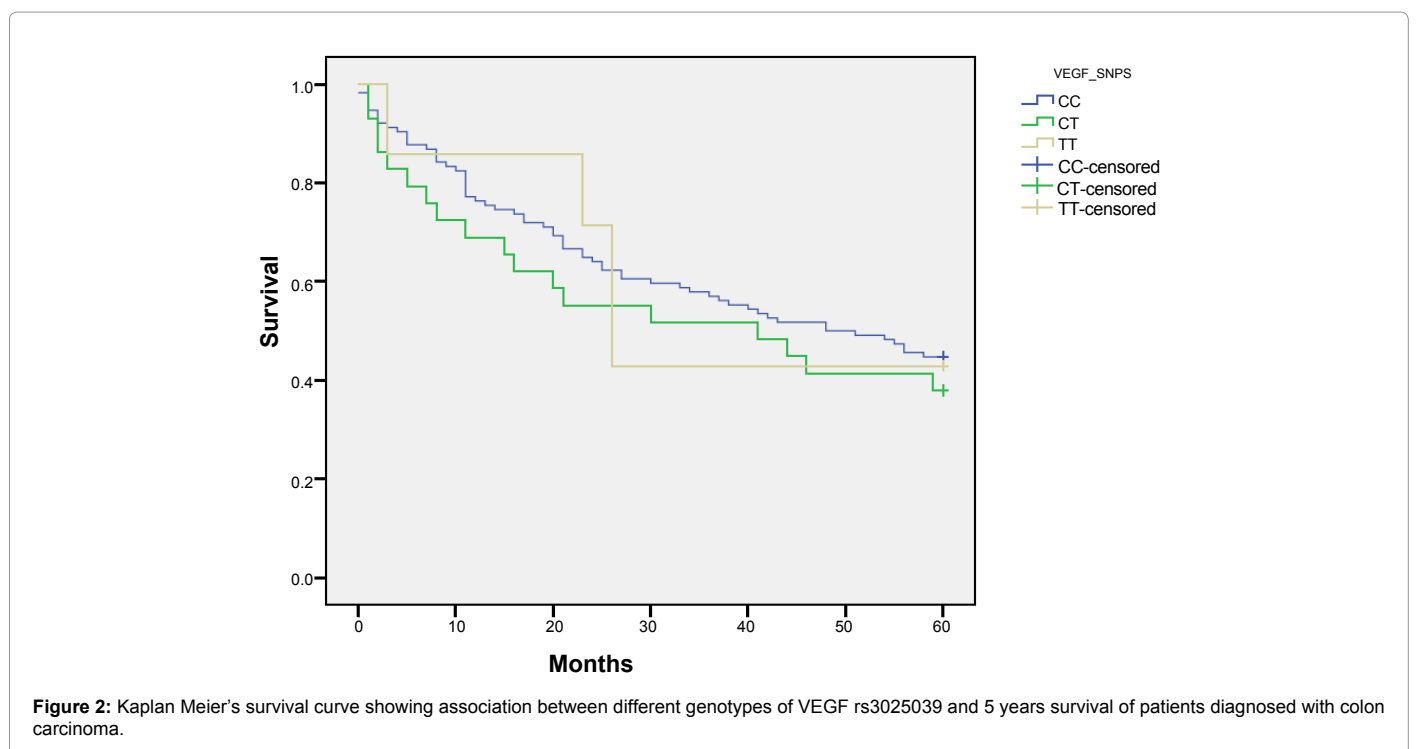
Genotypes	Low CI n (%age)	Medium CI n (%age)	High CI n (%age)	Total n (%age)	P value
CC	2 (5.0%)	10 (25.0%)	15 (37.5%)	27 (67.5%)	0.482
TC	0 (0.0%)	5 (12.5%)	6 (15.01%)	11 (27.5%)	
TT	0 (0.0%)	2 (5.0%)	0 (0.0%)	2 (5.0%)	
n=number					

Table 4: Complexity index association between different genotypes of VEGF rs3025039 polymorphism and growth pattern of tumors.

Parameters		CC n (%age)	TC n (%age)	TT n (%age)	P value
Age	Age 1	31 (20.7%)	9 (6.0%)	2 (1.3%)	0.886
	Age 2	83 (55.3%)	20 (13.3%)	5 (3.3%)	
Gender	Male	50 (33.3%)	15 (10.0%)	3 (2.0%)	0.783
	Female	64 (42.7)	14 (9.3%)	4 (2.7%)	
Tumor wall penetration T	T1	3 (3.1%)	0 (0.0%9	0 (0.0%)	0.936
	T2	12 (12.5%)	2 (2.1%)	0 (0.0%)	
	T3	55 (57.3%)	13 (13.5%)	4 (4.2%)	
	T4	5 (5.2%)	2 (2.1%)	0 (0.0%)	
Lymph node metastasis N	N0	47 (49.0%)	9 (9.4%)	3 (3.1%)	0.733
	N1	14 (14.6%)	5 (5.2%)	0 (0.0%)	
	N2	14 (14.6%)	3 (3.1%)	1 (1.0%)	
Systemic metastasis	Mx	74 (77.1%)	17 (17.7%9	4 (4.2%)	1
	M1	1 (1.0%)	0 (0.0%)	0 (0.0%)	
Localization	Right colon	54 (73.0%9	12 (16.2%)	3 (4.1%)	0.1
	Left colon	2 (2.7%)	2 (2.7%)	1 (1.4%)	
Differentiation	Low	15 (10.3%)	6 (4.1%)	3 (2.1%)	0.165
	Medium	76 (52.4%)	15 (10.3%)	3 (2.1%)	
	High	19 (13.01%)	7 (4.8%)	1 (0.7%)	
Duke,s stages	A	13 (9.0%)	4 (2.8%)	0 (0.0%)	0.976
	B	55 (37.9%)	13 (9.0%)	4 (2.8%)	
	C	37 (25.5%)	11 (7.6%9	3 (2.1%)	
	D	4 (2.8%)	1 (0.7%)	0 (0.0%)	
n=number					

Table 5: Genotypes of VEGF rs3025039 polymorphism and their association with different clinico-pathological parameters of the patients diagnosed with colon carcinoma.

of malignant diseases and particularly CRC is multifaceted [3,37,38], it seems that VEGF polymorphism and tumor growth pattern are not associated but could be two independent prognostic markers in colon carcinoma.

Furthermore, we investigated whether VEGF polymorphism has any effect on clinico-pathological parameters like age, gender, tumor penetration, lymph node metastasis, systemic metastasis, Duke's stages, localization and differentiation of tumor. There was no significant relationship between studied polymorphism and clinico-pathological parameters of the patients (p>0.05). Similar results were reported by other investigators which indicates that VEGF rs3025039 polymorphism has no significant effect on these parameters [33,34].

Conclusively, our study demonstrated that the TC genotype of VEGF rs3025039 polymorphism might be a protective factor and that VEGF polymorphism and tumor growth pattern are two independent prognostic markers in colon carcinoma. Further knowledge about the functionality of VEGF rs3025039 polymorphism is essential since it could lead to a better understanding of the tumor biology, behaviour and more importantly, the pharmacogenetic impact of these polymorphisms. Being an important target in anticancer therapy, information about VEGF polymorphism will be of great assistance for clinicians to tailor therapies in an individual manner.

Acknowledgement

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