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Original article

Vascular endothelial growth factor and endoglin (CD-105) in gastric cancer

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Abstract

Background. **Vascular endothelial growth factor (VEGF) overexpression has been associated with advanced stage and poor survival in several cancers. Additionally, CD-105 (endoglin) was proposed as a marker of neovascularization in solid malignancies. The aim of the present study was to (1) evaluate the VEGF and CD-105 expression in gastric carcinoma, (2) determine the role of *VEGF* gene sequence variations in VEGF expression in gastric carcinoma, and (3) correlate the results of VEGF and CD-105 expression with other standard prognostic parameters, such as size, grade, stage of the disease, metastases, and patient survival.**

Methods. **VEGF and CD-105 expression were evaluated in 100 unrelated gastric cancer patients using immunohistochemistry. For the genotyping, DNA was isolated from the blood of the gastric cancer patients and from 100 healthy individuals. The genotyping was performed by polymerase chain–restriction fragment length polymorphism analysis.**

Results. **VEGF protein was strongly expressed in the cytoplasm of 36% of the gastric carcinoma samples tested. In all cases, high VEGF expression was accompanied with high endoglin expression. Our results revealed no statistical significant association of any *VEGF* gene polymorphism with the VEGF and endoglin expression. The correlation of VEGF/CD-105 expression with the clinicopathological parameters of gastric cancer showed that the high expression of VEGF/CD015 was correlated only with lymph node metastasis ($P = 0.028$). The Kaplan-Meier survival curves have shown a clear association of overall survival after diagnosis of gastric cancer with high VEGF, as well as high CD-105 expression.**

Conclusion. **Our results support that VEGF and CD-105 are closely relevant to lymph node metastasis and act as two valuable indicators of prognosis.**

Key words VEGF · CD-105 · Gastric cancer · Prognosis

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Introduction

Angiogenesis, the formation of new blood vessels in and adjacent to a tumor, is essential procedure for tumor growth, as it supplies the tumor with nutrients and oxygen, eliminates the metabolic waste products of tumor cells, generates paracrine stimuli, and provides potential routes for tumor dissemination [1,2]. Additionally, neoangiogenesis contributes to metastasis because newly formed tumor vessels have less basement membrane material and fewer intercellular junctional complexes, which results in increased permeability and provides a route of exit to tumor cells into the circulation [3]. Angiogenesis has been proposed as a prognostic marker in a variety of human malignancies, including gastrointestinal neoplasms [4–7].

One of the most important angiogenic factors is vascular endothelial growth factor (VEGF). VEGF is a secreted homodimeric glycoprotein with several protein variants resulting from alternative mRNA splicing that can act as an endothelial cell mitogen and a modulator of changes in vascular permeability [8]. Interestingly, in vitro and in vivo experiments have shown that increased VEGF expression is associated with tumor growth and metastasis, whereas the inhibition of VEGF expression results in suppression of tumor growth and tumor-induced neoangiogenesis [9]. It is well known that several single nucleotide polymorphisms (SNPs) in the *VEGF* gene have been reported to affect the expression of the gene [10,11].

Endoglin (CD-105) is a receptor for transforming growth factor-β1 molecule, which binds preferentially to the activated endothelial cells that participate in tumor angiogenesis, with weak or negative expression in vascular endothelium of normal tissues [12]. Recently, several studies have indicated that endoglin is

a more specific and sensitive microvessel marker than other commonly used panedothelial antibodies in cancers of the cervix, colon, endometrium, and breast [12–15].

The aim of this study was to (1) evaluate the VEGF and CD-105 expression in gastric carcinoma, (2) determine the role of *VEGF* gene sequence variations in VEGF expression in gastric carcinoma, and (3) correlate the results of VEGF and CD-105 expression with other standard prognostic parameters, such as size, grade, stage of the disease, metastases, and patient survival.

Material and methods

Subjects

A total of 100 unrelated gastric cancer patients together with ethnic-, sex-, and age-matched 100 healthy individuals were used in the study. All patients and controls were born in and have been living in Greece. All patients gave their informed consent, and the hospital review board approved the study. The patients were followed up until June 2005 or death. The duration of follow-up (median ± SD and range) were 32.57 ± 29.57 months and 1–120 months, respectively. Altogether, 57 patients died during the follow-up. The characteristics of the gastric cancer patients at diagnosis are presented in Table 1.

Table 1. Histopathological characteristics of the Greek gastric cancer samples at diagnosis

Characteristic	Gastric cancer patients (no.)
Age at diagnosis (years), ±SD	65.0 ± 12.3
Male/female	58/42
Lymph node metastasis	
Negative	24
Positive	76
Other metastasis	
Negative	65
Positive	35
Stage at diagnosis	
I	18
II	12
III	45
IV	25
Histological grade	
Grade 1	14
Grade 2	34
Grade 3	52
Tumor size	
≤5 cm	52
>5 cm	48
Lauren classification	
Intestinal	47
Diffuse	53
Gross appearance	
Polypoid protrusion	13
Circumscribed excavation	4
Induration + ulceration	75
Diffuse thickening	8

DNA isolation and genotyping assays

DNA was extracted from peripheral blood using the NucleoSpin Blood Kit (Macherey-Nagel, Duren, Germany). Patients and control subjects were genotyped for the –2578C/A, –1154G/A, –634G/C, and +936C/T polymorphisms in the VEGF gene, as defined by Koukourakis et al. [16]. Synthesis of the appropriately sized polymerase chain reaction (PCR) products was visualized by agarose gel electrophoresis. The mutations were confirmed by sequencing analysis using a Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and an ABI 377 automated sequencer. As negative control of the PCR amplifications, we used distilled water instead of genomic DNA and confirmed the fidelity of the reactions.

Immunohistochemistry

Tumorous sections were immunohistochemically assessed for VEGF expression using the VG1 monoclonal antibody (recognizing the 121, 165, and 189 isoforms of VEGF) (Abcam, Cambridge, UK). Briefly, sections were dewaxed and incubated in 0.5% H_2O_2 in methanol for 30 min. After microwaving and washing in phosphate-buffered saline (PBS), sections were incubated with the primary antibody (1:4) for 90 min. After washing in PBS for 5 min, sections were incubated with goat anti-mouse immunoglobulins (1:200) for 30 min (Dako, Ely, UK), washed again with PBS, and incubated with rabbit anti-goat immunoglobulins (1:100) for 30 min. The peroxidase reaction was developed using diaminobenzidine (Sigma Fast tablets) as chromogen and sections were counterstained with hematoxylin. The number of cancer cells with VEGF cytoplasmic expression was assessed in all optical fields, and the median value was used to characterize each case. Carcinomas with strong VEGF expression in more than 50% of cancer cells were considered as being of high VEGF reactivity.

Endoglin staining was carried out using the anti-CD-105 monoclonal antibody (SN6h; Diagnostic Biosystems, Pleasanton, CA, USA). Briefly, sections were dewaxed in xylene, rehydrated, and pretreated with 3% H_2O_2 in PBS to block endogenous peroxidase activity. Immunohistochemical staining was performed with labeled streptavidin-biotin immunoperoxidase method (Super Sensitive IHC kit Detection Systems; BioGenex, San Ramon, CA, USA).To enhance immunostaining of

CD-105, proteinase K is used for proteolytic digestion, to expose hidden epitopes of the tissue section. Nonspecific binding was blocked using PBA (protein blocking agent, Power Block) for 5–10 min at room temperature. Slides were incubated for overnight at 4 °C with the anti-CD-105 monoclonal antibody (1:50). The samples were washed with PBS, stained with the biotinylated secondary antibody for 30 min, and washed again with PBS. Slides were then incubated with horseradish peroxidase-conjugated streptavidin for 20 min and washed again with PBS. The antigen-antibody reaction was visualized using diaminobenzidine (Biogenex) as chromogen, and sections were counterstained with hematoxylin. The number of cancer cells with CD-105 cytoplasmic expression was assessed in all optical fields, and the median value was used to characterize each case. Carcinomas with strong CD-105 expression in more than 50% of cancer cells were considered to have high CD-105 reactivity.

Assessments were performed by two independent pathologists (P.K., A.L.) who were blinded to the patients and the VEGF polymorphism data. Interobserver variability was minimal ($P < 0.01$). Discrepancies were observed in only three cases and were resolved on the conference microscope.

Statistics

Statistical analysis and graphs were performed using the GraphPad Prism 4.0 and the Instat 3.0 packages (GraphPad Software, San Diego, CA, USA). The frequency and susceptibilities of polymorphisms were compared to the chi-squared test. Odds ratios (OR) were calculated with the corresponding chi-squared distribution test and 95% confidence intervals (CI 95%). The P values obtained were two-tailed, and a strong association (significance) was assumed below 0.01. At $P > 0.05$, associations were assumed not significant. The Hardy-Weinberg equilibrium was verified by calculation of expected frequencies and numbers; and significance testing was based on the 1 d.f. χ^2. The effect of various variables on outcome was investigated by multivariate analysis using the Cox proportional hazards model. The survival curves were made using the Kaplan-Meier method, and comparison was with the log-rank test.

Results

VEGF protein was strongly expressed in the cytoplasm of 36 of 100 (36%) gastric carcinoma samples tested, with a median expression of 48% (range 10%–96%). Using a 50% cutoff point (strong staining in ≥50% of cancer cells), tumors were divided in two groups: (1) negative/low VEGF reactivity (64 cases) and (2) high (36 cases) VEGF reactivity. Regarding the endoglin (CD-105) protein, positive expression was observed in 30 of 100 (30%) gastric carcinoma samples examined, with a median expression of 50% (range 4%–95%). In this case also, using a 50% cutoff point (strong staining in ≥50% of cancer cells), tumors were divided in two groups: (1) negative/low CD-105 reactivity (70 cases) and (2) high (30 cases) CD-105 reactivity. It is important to note that all of the cases with high VEGF expression were accompanied with high endoglin expression (Fig. 1).

In a previous study in our laboratory [17] we had examined the effect of four common polymorphisms in the *VEGF* gene on gastric cancer development in the

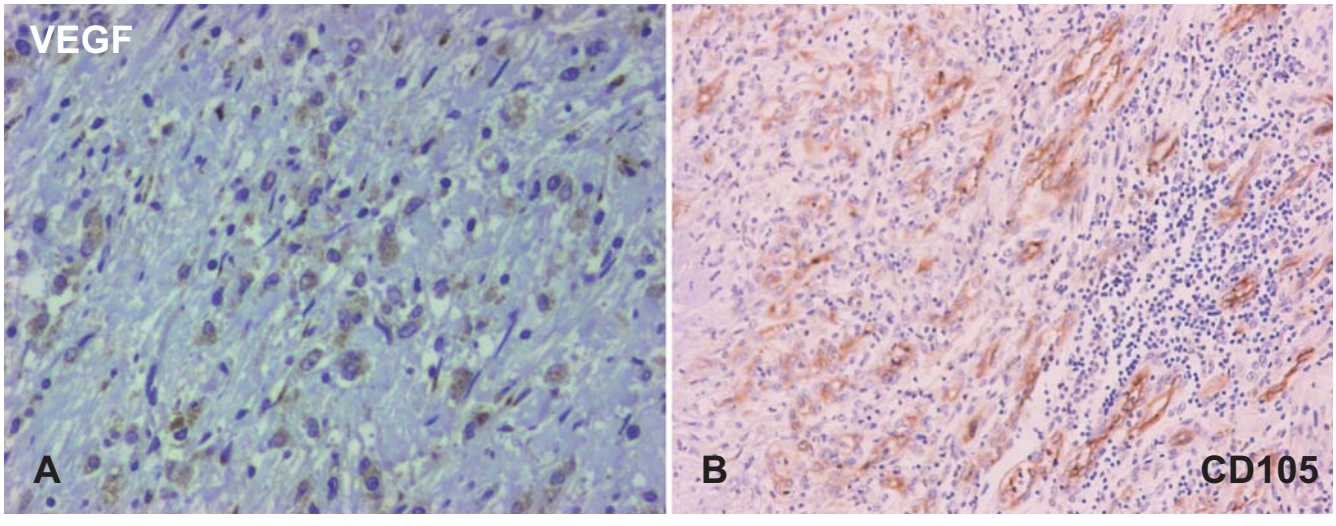

Fig. 1. Representative results of high immunostaining of VEGF (**A**) and CD-105 (**B**) in gastric carcinoma

Table 2. Allele and genotype distributions of the polymorphisms in the *VEGF* gene in a Greek population

Genotype/allele distributions	Cases (%)	Controls (%)	*P*; OR (95% CI)
–2578 C/A			
CC	32	21	1.00
CA	39	48	0.193; 0.69 (0.39–1.21)
AA	29	31	0.866; 0.95 (0.52–1.74)
A%	53.5	55	
–1154 G/A			
GG	45	42	1.00
GA	36	43	0.311; 0.75 (0.42–1.32)
AA	19	15	0.451; 1.33 (0.63–2.79)
A%	37	36.5	
–634 G/C			
GG	41	52	1.00
GC	40	39	0.885; 1.04 (0.59–1.84)
CC	19	9	0.042; 2.37 (1.02–5.54)
C%	39	28.5	
+936 C/T			
CC	41	51	1.00
CT	33	27	0.354; 1.33 (0.72–2.44)
TT	26	22	0.508; 1.25 (0.65–2.39)
T%	42.5	35.5	

Table 3. Cox proportional hazard estimation of overall survival

Covariant	B coefficient	SE	*P*	Relative Risk	95% CI for relative risk
Overall survival					
Disease status	0.907	0.181	<0.001	2.48	1.74–3.53
VEGF expression	0.365	0.354	0.300	1.44	0.72–2.88
CD-105 expression	–0.656	0.364	0.072	0.52	0.25–1.06

same patient group. The genotype distribution of all studied polymorphisms followed the Hardy-Weinberg equilibrium. The genotype and allele distribution among the gastric cancer cases and controls are shown in Table 2. Interestingly, the CC genotype of the –634G/C polymorphism was significantly overrepresented in gastric cancer patients compared to healthy individuals ($P = 0.042$). No significant differences in the remaining genotype frequencies between gastric cancer cases and controls were detected. In this study, we linked the VEGF and endoglin expression with the *VEGF* genotype. Our results revealed no statistically significant association of any *VEGF* polymorphism with VEGF or endoglin expression.

The correlation of VEGF/CD-105 expression with the clinicopathological parameters of gastric cancer showed that the high expression of VEGF/CD015 was correlated only with lymph node metastasis ($P = 0.028$).

During the study period, there were 57 deaths among the patients in the study. There were no differences with respect to sex, age, or location of the tumor for patients with long and short survival times. Nevertheless, the univariate analysis demonstrated a significant association between advanced stages of disease (III and IV) and decreased overall survival ($P < 0.001$).

The Kaplan-Meier survival curves by VEGF and CD-105 expression (Fig. 2) showed a clear association of overall survival after diagnosis of gastric cancer with high VEGF and high CD-105 expression. The 10-year survival rate was 27.77% for patients with high VEGF/CD-105-expressing tumors, which was significantly lower than the rate among patients with lower VEGF/CD-105-expressing tumors (51.56%, $P = 0.034$). The multivariate analysis disease status and VEGF/CD-105 expression emerged as not independent variables of adverse prognostic significance (Table 3).

Discussion

Tumor angiogenesis and its clinical importance have been studied in a variety of human neoplasms. Several investigators have shown a significant correlation between VEGF expression and neoagiogenesis in various malignancies [18,19]. Additionally, a variety of studies support a significant correlation among VEGF expression and microvessel (MV) count [19,20], presence of

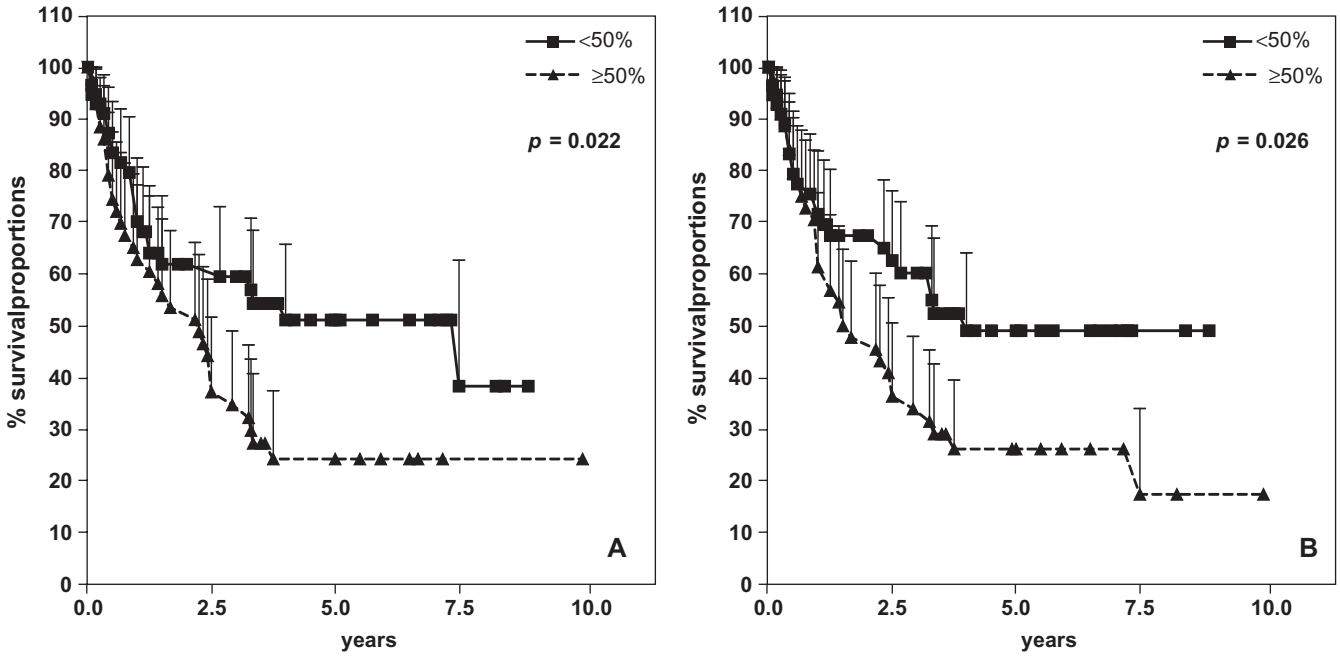

Fig. 2. Overall survival of patients after diagnosis of gastric cancer by *VEGF* (**A**) and CD-105 (**B**) expression

lymph nodes metastases [20–22], depth of tumor invasion, and the presence of distant metastasis [19–22]. Some studies also demonstrated a relation between VEGF expression and patient outcome as an independent prognostic factor [22,23].

In the present study we observed that VEGF expression was closely related with endoglin (CD-105) expression, which is a sensitive microvessel marker, supporting that VEGF could induce formation of new blood vessels. CD-105 has already been proven to serve as an important marker of survival in patients with solid tumors of various histotypes [24]. In breast carcinomas, the CD-105 positive tumors correlated significantly with poor overall survival [25]. Colorectal cancer patients with CD-105 expression above the median showed the worst prognosis, and the 5-year survival rate of patients with endometrial cancer was higher if they featured low endoglin tissue level expression [26,27]. Little if any evidence exists on the clinical significance of endoglin in gastric carcinomas. Its biological role is not fully elucidated yet, either. Yu et al. have shown a close relation between CD-105 MVD and transforming growth factor-β1 (TGFβ1) expression in gastric cancer tissue specimens [28]. The biological rational of this correlation lies mainly in the fact that CD-105 is one of the receptors of TGFβ1 [28]. On the other hand, TGFβ1 is a potent inhibitor of endothelial cell proliferation and migration in vitro and an angiogenesis promoter in vivo [28].

The VEGF and CD-105 expression may play an important role in tumor biological behavior, progression, and prognosis. Indeed, a strong correlation was found between VEGF and CD-105 expression and lymph node metastasis in gastric carcinoma. The strong expression of VEGF and CD-105 in our study indicated a poor prognosis. Our findings remain in line with many studies showing the independent prognostic value of VEGF expression [7,29]. In addition to endoglin's clinical significance in other malignancies, our results support the value of this new angiogenesis marker as an independent prognostic marker in gastric carcinomas as well. The expression of VEGF was positively related to endoglin expression in our research. The latter provides further evidence supporting the causality of VEGF and angiogenesis stimulation, leading to tumor growth, infiltration, and metastasis. The newborn vessels have important effects on the aggressive phenotype and the metastatic potential of tumors. This is due to their unique structural and functional features (i.e., their inability to differentiate to arteries or veins, lack of smooth muscle, inability to contract, prone to spontaneous thrombosis or hemorrhage) [28]. Under these circumstances, carcinoma cells can invade new vessels more easily and can be carried to other organs to form a metastastic focus.

In contrast to other studies that demonstrated that specific genetic polymorphism in the VEGF gene influences the levels of VEGF expression [10,11,16], our results did not reveal any statistically significant association between the particular genotype of the VEGF gene and VEGF and endoglin expression levels. The VEGF SNPs may carry a clinical and biological significance

irrelevant to VEGF tissue expression. The relations between the SNPs and the tumors' behavior remains largely obscure. Certain SNPs might reflect a VEGF gene functional alteration, either toward the activation or the repression side, that requires synergistic expression or action of cofactor molecules [17].

VEGF and CD-105 are closely relevant to lymph node metastasis and act as valuable indicators of the prognosis. Overexpression of CD-105 on proliferating endothelial cells of the tumor vasculature suggests that CD-105 might represent a good target for gastric cancer immunoscintigraphy [24]. Based on current animal model experimental data, the use of this immune marker may expand to immuno-guided surgical planning of the extent of gastric and other alimentary tract cancer resections [25]. Additionally, targeting of CD-105, as a therapeutic antiangiogenic approach in cancer, has been extensively investigated in severe combined immunodeficiency mice bearing human breast tumors [24]. Our histological and molecular data on VEGF, with its polymorphisms and important neoangiogenesis markers such as endoglin, may provide an important basis for future therapeutic trials with antiangiogenic therapeutic regimens.

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