Exp Oncol 2015
37, 3, 213–217

Experimental ONCOLOGY

ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN NON-SMALL CELL LUNG CANCER PATHOGENESIS

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The angiogenesis is an important process in the pathogenesis of malignancies. It is regulated by various growth factors, with the vascular endothelial growth factor (VEGF) playing the central role. *The aim* of the present study was to evaluate possible associations of functional *VEGF* −2578C>A, −634G>C, and +936C>T polymorphisms with the risk for occurrence and progression of non-small cell lung cancer (NSCLC) in patients living in Republic of Belarus. *Materials and Methods:* A total of 202 patients (147 males and 55 females) diagnosed as having the NSCLC. The control group consisted of 336 individuals (245 males and 91 females) without an oncopathology. The total DNA was isolated from peripheral blood. We investigated the single nucleotide polymorphisms of *VEGF* (rs 2010963), (rs 699947), (rs 3025039). The genotyping was performed by PCR-RFLP analysis. *Results*: Our results revealed a marginally significant association of the −2578CC genotype (p=0.002) with a greater degree of tumor spread (T2–T4). Heterozygous genotypes −2578CA and +936CT carriers were included into the follow-up group significantly more often (p=0.021 and p=0.012, respectively). Our study demonstrate that *VEGF* −2578A/C and +936C/T polymorphisms are among the factors determining the individual peculiarities of NSCLC course in this population and can be used for clarifying the prognosis of the disease.
***Key Words*: vascular endothelial growth factor, polymorphism, non-small cell lung cancer.**

The angiogenesis is an important process in the pathogenesis of malignancies. It is regulated by various growth factors, with the vascular endothelial growth factor (VEGF-A or VEGF) playing the central role [1]. The VEGF gene expression is closely related to the degree of vascularization and the prognosis for the occurrence of numerous solid tumors and is considered to be a predictor of resistance to the chemo- and radiotherapy [2]. An elevated VEGF expression is associated with tumor growth and metastatic process, while the inhibited VEGF expression results in suppressed tumor growth [3].

The *VEGF* gene also triggers the activation of the protease cascade involved in the degradation of extracellular matrix, suppressing apoptosis, stimulates the endothelial cells survival, increases vascular permeability, inhibits the dendritic cells differentiation, regulates the hexose transport in endothelial cells as well as activates tissue factors and monocytes migration [1]. Clinical studies have shown that the high level of VEGF expression and, respectively, the increased number of microvessels in the tumor correlate with the disease stage and the unfavorable prognosis for many tumor types, including the non-small cell lung cancer (NSCLC) [4–8].

The *VEGF* gene is located on the short arm of chromosome 6 (6p21.3) and consists of eight exons and seven introns [7]. VEGF is a diametric glycoprotein, acting via tyrosine kinase receptors VEGFR1 and VEGFR2 located predominantly on endothelial cells [9].

Polymorphic sites of *VEGF* gene determining the level of VEGF production are found in the promoter — 5′-untranslated region (5′-UTR) as well as in 5′- and 3′-untranslated regions of the gene (3′-UTR).

The VEGF gene polymorphism is associated with differentiated VEGF expression and protein production. *VEGF*-2578C/C, −634 C/-genotypes are related to high level of VEGF expression [5, 9–12], while +936T allele correlates with low VEGF expression and its low levels in blood plasma [13, 14].

Recent studies show that individual polymorphic variants of *VEGF* influence the development of certain cancers. In particular, *VEGF* C-634G, C-2578A, C+936T polymorphisms are associated with an increased lung cancer risk in Asian population [15]. Based on the above-mentioned, it can be assumed that these single nucleotide substitutions of the *VEGF* gene related to angiogenesis can affect the risk of tumour occurrence and progression as well as the patients' survival.

The aim of the present study was to evaluate possible associations of functional *VEGF* −2578C>A, −634G>C, and +936C>T polymorphisms with the risk for occurrence and progression of NSCLC in patients living in the territory of the Republic of Belarus.

MATERIALS AND METHODS

202 patients (147 males and 55 females) diagnosed as having the NSCLC and treated at the Minsk City Oncology Dispensary during the period from 2003 to 2012 were included in the study. The control group consisted of 336 individuals (245 males and 91 females) without an oncopathology who were age-, gender- and comorbidity matched with NSCLC pa-

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Abbreviations used: 3′-UTR – 3′-untranslated region; 5′-UTR – 5′-untranslated region; NSCLC – non-small cell lung cancer; VEGF – vascular endothelial growth factor.

tients. Clinical characteristics of patients with NSCLC and controls are presented in Table 1.

Table 1. Characteristics of study population

Characteristics	Patients with NSCLC (n = 202) n (%)	Controls (n = 336) n (%)
Gender:		
female	55 (27.2)	91 (27.1)
male	147 (72.8)	245 (72.9)
Smoking status:		
smokes	67 (33.2)	201 (59.8)
does not smoke	119 (58.9)	135 (40.2)
no information	16 (7.9)	–
Stage:		
I	108 (53.5)	–
II	27 (13.4)	–
III	55 (27.2)	–
IV	12 (5.9)	–
Histology:		
squamous-cell carcinoma	106 (52.5)	–
adenocarcinoma	96 (47.5)	–
Surgery:		
lobectomy/bilobectomy	121 (65.1)	–
pneumonectomy	42 (22.6)	–
lung resection	13 (7.0)	–
biopsy	10 (5.4)	–
Therapy:		
chemotherapy	65 (32.2)	–
radiotherapy	39 (19.3)	–
no therapy	98 (48.5)	–

The study was performed in compliance with the principles of voluntary participation and confidentiality, in accordance with the questioning of patients and the approval from the local Ethics Committee to study tissue samples and biological fluids.

The diagnosis of lung cancer in patients has been established on the basis of clinical signs of the disease, history data, bronchoscopy, X-ray examination and computed tomography, cytomorphology of sputum and tumour tissue biopsies. In the group of NSCLC patients, the mean age was 61.6±0.6, in the control group — 61.6±0.8 years.

All cases of lung malignancies have been identified according to the International Classification TNM/pTNM (7th edition, 2009). The histological type of lung carcinoma was determined according to the WHO histological criteria (3rd edition, 1999). The group of NSCLC included the most common neoplasms — squamous-cell carcinoma and adenocarcinoma. The treatment was mostly surgical: lobectomy — 65.1%, pneumonectomy — 22.6%, lung resection — 7.0%. Only a biopsy was performed in 5.4% of cases, and in 7.9% of patients the operation was not done because of the process spread or severe comorbidity.

The total DNA from the peripheral blood was isolated using the Mathew method [16]. *VEGF* C-634G (rs 2010963), C-2578A (rs 699947), C+936T (rs 3025039) polymorphisms were genotyped by the polymerase chain reaction method and the restriction fragment length polymorphism analysis (PCR-RFLP analysis) using specific primers and restriction endonucleases [15, 17]. Primers for the analysis were synthesized by "Primetekh", Minsk. The reagents for PCR and PCR-RFLP were manufactured by "Fermentas", Vilnius.

Statistical analysis. Statistical analyses were performed using Excel and Statistica 7.0. When comparing genotype frequencies, Pearson's chi-squared test (χ^2) was used. The association between the genotypes and the disease course was assessed by the value of odds ratio (OR).

RESULTS AND DISCUSSION

An elevated VEGF expression was revealed while studying the variety of malignancies: cancers of the colon, rectum, liver, lung, thyroid, digestive tract; breast, kidney and bladder adenocarcinoma; ovary and uterus carcinoma; angiosarcoma, multiform glioblastoma [18–21]. In turn, the level of VEGF expression depends largely on the polymorphic variants of the gene.

Therefore, the first phase of our work was to analyses the association between *VEGF* C-634G, C-2578A and C+936T polymorphisms and the risk of NSCLC.

The analysis of data on *VEGF* genotyping in patients with NSCLC and controls is presented in Table 2.

Frequency distribution of alleles and genotypes of functionally significant *VEGF* polymorphisms in the population of patients living in the territory of the Republic of Belarus was close to the occurrence of the same polymorphisms in the control group. Statistically significant differences in the frequency of the studied genotypes between the group of patients with NSCLC and the control group were not

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found. Similar results on the frequency of the studied *VEGF* single nucleotide substitutions were obtained for the populations in Germany and Sweden.

Based on the results, it can be assumed that there is no connection between the studied *VEGF* polymorphisms and the risk of NSCLC in this population.

Since *VEGF* is a major mediator of angiogenesis, it can be supposed that *VEGF* C-634G, C-2578A and C+936T polymorphisms will rather affect the phenotype and tumor biological behavior than the risk for tumor occurrence.

The next stage of our study was to find the relationship between the polymorphisms under study and the clinical course of this disease. The comparative analysis of genotypes distribution of three functionally significant *VEGF* polymorphisms and tumor size, metastasis and clinical outcomes in the group of patients with NSCLC has been carried out.

VEGF triggers the neoplastic angiogenesis, resulting in increased microvascular density, and malignant tissue receives more nutrients [23, 24]. VEGF secretion by tumor cells leads to the synthesis of proangiogenic factors. The newly formed vessels begin to supply malignant tissue with oxygen and nutrients; the tumor is growing and producing more VEGF. VEGF increases the level of VEGFR2 receptor expression by endotheliocytes of tumor microvessels, thus stimulating the cell growth and endothelial cells proliferation [25].

Structurally and functionally the neoplastic vessels differ from the normal ones, with high permeability, chaotic branching, multiple loops, weaves, dead-end branches, lack of structured vasculature being typical for them [2]. Chaotic arrangement of tumor vessels results in uneven oxygen supply to the surrounding tissues, formation of local hypoxic foci, and is followed by tumor resistance to the radio-and chemotherapy.

With an elevated VEGF expression, the vascular permeability increases leading to a higher interstitial and intratumoural pressure, facilitating the penetration of tumor cells into the bloodstream [26].

The relationship between the tumor angiogenesis level, tumor size and metastatic process is confirmed by the correlation between the course of the disease and microvascular density of the primary tumor [27–32]. Different *VEGF* polymorphisms are associated with lymphogenic and hematogenous metastases in a variety of malignancies [28, 29].

The performed analysis of the relationships between three *VEGF* gene polymorphisms and tumor size (Table 3) has shown that genotype –2578CC carriers displayed a greater degree of tumor spread (T2–4) significantly more frequently ($p = 0,002$) compared to the primary focus spread (T1). On the contrary, the carriers of –2578CA genotype more often presented with small non-invasive cancer (T1). However, in this group of NSCLC patients, no statistically significant associations between the studied polymorphic allele variants and regional and/or distant metastases were found (Table 4).

The *in vitro* studies conducted by M. Mohammadi and M.C. Shahbazi has shown that -2578C allele correlated with higher VEGF expression compared to A allele [30, 31]. Similar results were obtained by M. Perrot-Applanat who proved that –2578C allele was associated with an elevated VEGF expression and pointed to the possible involvement of VEGF proteins in the autocrine regulation of the tumor growth [32]. Perhaps, this is likely to explain our findings, where the homozygous carriers (–2578CC) had higher degree of the tumor spread. The lower level of VEGF expression in the presence of –2578A allele seems to have a protective effect in heterozygous carriers (–2578CA) associated with a lesser degree of the neoplasm spread.

In the population under study, a significant relationship between the –2578CA variant and the degree of spread of the primary tumor as well as the outcome of the disease was detected (Table 5). Heterozy-

Table 2. Frequency distribution of polymorphic variants of VEGF in patients with NSCLC and control groups in different populations

Genotype	Belarus (original data)			Germany [22]			Sweden [22]		
	Patients	Controls	OR (95% CI)	Patients	Controls	OR (95% CI)	Patients	Controls	OR (95% CI)
VEGF (G634C)	N=186; n (%)	N=364; n (%)		N, %	N, %		N=936; n (%)	N=941; n (%)	
GG	83 (44.6)	186 (51.1)	0.77 (0.54–1.10)	–	–	–	488 (52.1)	492 (52.3)	1.00
GC	88 (47.3)	154 (42.3)	1.22 (0.86–1.75)	–	–	–	363 (38.8)	367 (39.0)	1.00 (0.82–1.21)
CC	15 (8.1)	24 (6.6)	1.24 (0.64–2.43)	–	–	–	85 (9.1)	82 (8.7)	1.05 (0.74–1.47)
G allele	254 (68.3)	526 (72.3)	0.83 (0.63–1.09)	–	–	–	1339 (71.5)	1351 (72.0)	0.99 (0.86–1.14)
C allele	118 (31.8)	202 (27.7)	1.21 (0.92–1.59)	–	–	–	533 (28.5)	531 (28.0)	1.01 (0.86–1.17)
VEGF (C2578A)	N=162; n (%)	N=360; n (%)		N=153; n (%)	N=162; n (%)		N=939; n (%)	N=940; n (%)	
CC	41 (25.3)	83 (23.0)	1.13 (0.74–1.74)	44 (28.8)	50 (30.9)	1.00	258 (27.5)	257 (27.3)	1.00
CA	90 (55.6)	186 (51.7)	1.17 (0.81–1.70)	75 (49.0)	72 (44.4)	1.18 (0.68–2.06)	449 (47.8)	451 (48.0)	0.99 (0.79–1.24)
AA	31 (19.1)	91 (25.3)	0.70 (0.44–1.11)	34 (22.2)	40 (24.7)	0.97 (0.50–1.86)	232 (24.7)	232 (24.7)	1.00 (0.82–1.23)
C allele	172 (53.1)	352 (48.9)	1.18 (0.91–1.54)	163 (53.3)	172 (53.1)	1.01 (0.74–1.38)	965 (51.4)	965 (51.2)	0.78 (0.68–0.89)
A allele	152 (46.9)	368 (51.1)	0.85 (0.65–1.10)	143 (46.7)	152 (46.9)	0.99 (0.73–1.36)	913 (48.6)	915 (48.8)	1.05 (0.93–1.20)
VEGF (C936T)	N=161; n (%)	N=360; n (%)		N=153; n (%)	N=163; n (%)		N=924; n (%)	N=934; n (%)	
CC	115 (71.4)	267 (74.2)	0.87 (0.57–1.32)	120 (78.4)	128 (78.5)	1.00	708 (76.6)	720 (77.1)	1.00
CT	38 (23.6)	80 (22.2)	1.08 (0.70–1.68)	31 (20.3)	31 (19.0)	1.07 (0.59–0.93)	204 (22.1)	203 (21.7)	1.02 (0.81–1.28)
TT	8 (5.0)	13 (3.6)	1.40 (0.57–3.44)	2 (1.3)	4 (2.5)	0.53 (0.07–3.46)	12 (1.3)	11 (1.2)	1.11 (0.45–2.71)
C allele	268 (83.2)	614 (85.3)	0.86 (0.60–1.23)	271 (59.8)	287 (88.0)	0.20 (0.14–0.30)	1620 (87.7)	1643 (88.0)	0.97 (0.80–1.18)
T allele	54 (16.8)	106 (14.7)	1.17 (0.82–1.67)	182 (40.2)	39 (12.0)	4.94 (3.37–7.25)	228 (12.3)	225 (12.0)	1.03 (0.84–1.25)

Table 3. Distribution of frequencies of polymorphic variants of *VEGF* and tumor size in patients with NSCLC

Tumour size	N	G634C						N	C2578A						N	C936T					
		GG		GC		CC			CC		CA		AA			CC		CT		TT	
		n	%	n	%	n	%		n	%	n	%	n	%		n	%	n	%	n	%
T1	70	32	45.7	33	47.1	5	7.1	57	6	10.5*	40	70.2**	11	19.3	41	29	70.7	11	26.8	1	2.4

gous genotype –2578CA carriers were included into the follow-up group significantly more often (p = 0.021). It should be noted that a similar results was observed for another *VEGF* polymorphism — the heterozygous variant +936CT located in the 3′-UTR (p = 0.012). Moreover, C. Oliveira et al. have shown that heterozygous +936CT genotype was increasingly associated with a non-recurrent disease [11]. No associations between the *VEGF* –634G>C polymorphism and the survival of patients with NSCLC have been found.

It has been demonstrated that the polymorphism of a single nucleotide located in the promoter region or 3′- and 5′-UTR affects the protein expression at the transcriptional level [33]. Initially, the hypoxia induced factor (HIF-1) joins *VEGF* gene in the promoter region (–2578CA polymorphism) and increases its expression. Gene polymorphism in this region can weaken or strengthen this interaction, and, therefore, alter the VEGF expression (–2578CA) [34] that, in turn, will affect the formation and permeability of microvessels and, finally, the tumor size, metastasis and survival.

Based on the results of the study on the correlation between the polymorphisms and development of the disease in this population, it has been established that the carriers of –2578CC genotype displayed a greater degree of tumor spread (T2–4) significantly more often (p = 0.002) while the carriers of –2578CA genotype presented with small non-invasive cancers (T1) more frequently (p = 0.021). The carriers of –2578C/A genotype were significantly often observed (p = 0.021) in the "follow-up" group. Given that the high level of VEGF expression is related to an increased risk of a recurrent disease and shorter survival of subjects with different cancers [35], one may suggest the VEGF (–2578C/-) genotype to contribute to the high expression of the corresponding protein product in the development of NSCLC.

The mechanism of most associations between single nucleotide substitutions and clinical behavior of tumors is still largely unclear. It should be taken into account that 5′- and 3′-UTR contain key regulatory elements sensitive to hypoxia [36–38] and contribute to high variability of VEGF production [39]. For instance, VEGF-634G>C and –2578A>C polymorphisms in the 5′-UTR affect the efficiency of protein translation [33], and 936C>T polymorphism in the 3′-UTR influences the VEGF circulating plasma concentrations [13] and tumor tissue expression of VEGF [5]. This can explain the protective effect of the VEGF +936T allele in breast cancer metastasis (due to the reduced VEGF expression) [4, 11].

Thus, the results of our study demonstrate that VEGF –2578A/C and +936C/T polymorphisms are among the factors determining the individual peculiarities of NSCLC course in this population and can be used for clarifying the prognosis of the disease.

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