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Distribution of the C-460T polymorphism of the vascular endothelial growth factor gene in age-related macular degeneration

Rozkład genotypów polimorfizmu C-460T genu naczyniowo-śródbłonkowego czynnika wzrostu w zwyrodnieniu plamki związanym z wiekiem

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Summary: Purpose: To assess the association between genotypes and alleles of the C-460T polymorphism of the vascular endothelial growth factor (VEGF) gene and the risk of wet form of age-related macular degeneration (AMD).
Materials and methods: 100 patients with clinically diagnosed wet form of AMD and 104 healthy individuals were enrolled in this study. The patients were diagnosed by optical coherence tomography, fluorescein angiography and indocyanin green angiography. The allele-specific polymerase chain reaction was used to determine the genotypes of the C-460T polymorphism of the VEGF gene. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using a logistic regression model to assess the assoctiation betweeen genotypes of the C-460T polymorphism and AMD occurrence.
Results: A difference was observed in the genotype distributions between patients and controls. An association (OR 3.04, 95% CI 1.65-5.60) was found between wet form of AMD and the C/T genotype. On the other hand, the T/T genotype displayed the protective effect against the disease.
Conclusion: The C-460T polymorphism of the endothelial growth factor can be considered as a potential marker for the wet form of age-related macular degeneration.

Key words: age related macular degeneration, AMD, vascular endothelial growth factor, VEGF, genetic polymorphism.
Słowa kluczowe: zwyrodnienie plamki związane z wiekiem, AMD, naczyniowo-śródbłonkowy czynnik wzrostu, VEGF, polimorfizm genetyczny.

Introduction

Age-related macular degeneration (AMD) is the overall leading cause of visual loss and neovascularization seems to be primarily involved in the fatal outcome of the wet form of this disease (see 1 for review). The process of neovascularization is underlain by several angiogenic factors, including vascular endothelial growth factor, VEGF, which seems to be crucial for this process. Recent studies have documented the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients (2). These findings suggest a role for VEGF in the progression of AMD-related choroidal neovascularization, raising the possibility that VEGF inhibition may have therapeutic value for this condition. Accordingly, clinical trials with anti-VEGF antibodies and other VEGF inhibitors are currently ongoing (3).

VEGF or VEGF-A, is a member of a family of related proteins consisting of VEGF-A, VEGF-B, VEGFC, VEGF-D and Placenta Growth Factor. Along with Platelet-derived Growth Factor A and B (PDGF-A and PDGF-B) they form a protein superfamily. The *VEGF-A* pre-mRNA is alternatively spliced and is translated to give 5 polypeptide products of 121, 145, 165, 189 and 206 amino acids (4). VEGF-A is crucial to embryonic development and disruption of even one allele of the *VEGF-A* gene is lethal (5).

The expression of the *VEGF* gene is modulated by a variety of effectors including cytokines, lipopolysaccharide (LPS), hormones and hypoxia (6). Dysregulated VEGF expression is implicated in a number of disease pathologies. Increased VEGF expression resulting in inappropriate VEGF-induced angiogenesis is linked with tumour growth and metastasis, rheumatoid arthritis and diabetic retinopathy. Conversely, the ability to produce VEGF in response to hypoxia is linked to the development of collateral vessels and protection against myocardial disease.

In the eyes of healthy subjects, VEGF is present only in a minimal degree in endothelial cells of retinal and choroidal vessels, retinal pigment epithelium (RPE) and intravascular leukocytes of choroid and retina (7). Because VEGF is important for the process of neovascularization, which, in turn, is essential for wet form of AMD, the expression of the *VEGF* gene may be of great importance in the pathogenesis of this disease.

This expression can be modulated by the variability of the gene, which can take a form of polymorphism. Therefore, it is reasonable to search for the association between sequence variability of the *VEGF* gene and wet form of AMD. In the present work we checked the association between genotypes and alleles of the C-460T polymorphism of the *VEGF-A* gene and the risk of the occurrence of AMD in wet form.

Materials and methods

Patients

Blood samples were obtained from patients with wet form of AMD (n = 100) and healthy individuals (n = 104).

Medical history was obtained from all subjects. The patients underwent ophthalmic examination including best corrected visual acuity, intraocular pressure, slit-lamp examination, and fundus examination using non-contact and contact fundus lenses with a slit lamp.

Diagnosis of wet form AMD was confirmed by optical coherence tomography (OCT), fluorescein angiography (FA), and in some cases indocyanin green angiography (ICG). OCT evaluated retinal thickness, the presence of subretinal fliud and intraretinal oedema; angiography assessed the anatomical status of the retinal vessels, the presence of choroidal neovascularisaton (CNV) and leakage.

The OCT examinations were performed with Stratus OCT model 3000, software version 4.0. The FA and ICG examinations were completed with a Topcon TRC-50I IX fundus camera with the digital Image Net image system (ver. 2.14; Topcon Co., Tokyo, Japan).

The Local Ethic Committee approved the study and each patient gave a written consent.

Determination of the *VEGF* genotype

DNA was isolated from peripheral blood leukocytes by using proteinase K digestion and phenol/chloroform extraction. Genotypes of the C–460T polymorphism was determined by the allele specific PCR. Two forward primers annealed to the polymorphic site T: 5'-TGCGTGTGGGGTTGAGGGT-3' or C: 5'-TGCGTGTGGGGTTGAGGGC-3' and reverse primer: 5'-CCCGCCGCAATGAAGGGGA-3' were used. The PCR was carried out in a MJ Research, INC thermal cycler, model PTC-100 (Waltham, MA, USA), in a total volume of 25 μl, containing 50 ng genomic DNA, 10 pmol each primer (Sigma Proligo, Sigma-Aldrich, Mannheim, Germany), 200 mM each dATP, dCTP, dGTP and dTTP (Boeringer, Mannheim, Germany), 20 mM Tris-HCl (pH 8.4) 50 mM KCl, 2 mM $MgCl_2$ and 1 unit Taq polymerase. The thermal cycling conditions were 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 45 s at 61°C and 1 min at 72°C. PCR-amplified DNA was analyzed on a 3% agarose gel and visualized by ethidium bromide staining. Typical results of genotype analysis are displayed in Fig. 1.

Statistical analysis

The significance of the differences of observed alleles and genotypes between groups was tested using the chi- test-based analyses. The ORs and 95% CIs were calculated using a logistic regression model. The two-group comparisons of continuous data with normal distribution (verified with Shapiro-Wilk's test) were performed with t-test, the remianing data were analyzed with the non-parametric Mann-Whitney U test. ANOVA was used to identify parameters that would make significant differences when comparing more than two groups; Scheffe's test was then used as the post hoc multiple comparison test. Analyses were performed using STATISTICA 6.0 software (Statsoft, Tulsa, OK, USA).

Results and discussion

All the patients and controls were divided into three genotypes of the C–460T polymorphism of the *VEGF-A* gene promoter: C/C, C/T and T/T (Table 1). There was a significant

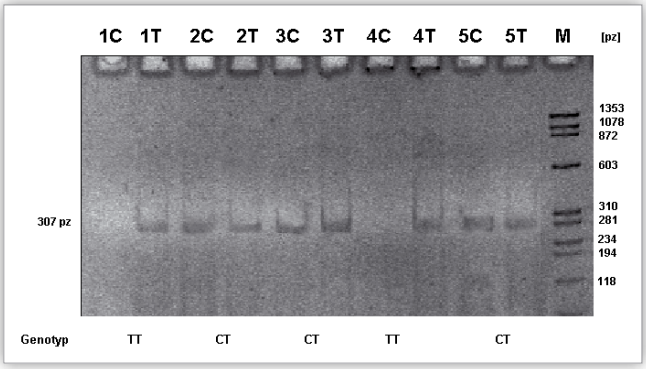

Fig. 1. Genotypes of the C/ T polymorphism at the – 460 bp *VEGF-A* promoter region determined by the allele specific PCR-detection (ASO-PCR) and analysed by 3% agarose gel electrophoresis, stained with ethidium bromide and viewed under UV light. Lane M displays molecular weight marker; the lanes show the results of amplification with primer specific either to the C allele or the T allele.

Ryc. 1. Genotypy polimorfizmu C/T w pozycji -460 obszaru promotorowego genu *VEGF-1* określone metodą allelo-specyficznej PCR i analizowane przez elektroforezę w 3% żelu agarozowym, barwienie bromkiem etydyny i naświetlanie UV. Ścieżka M zawiera marker mas cząsteczkowych, pozostałe ścieżki – wyniki amplifikacji ze starterami specyficznymi do allelu C lub T.

Genotype or Allele	AMD (n = 100)		Controls (n = 104)		OR (95% CI)
	Number	Frequency	Number	Frequency	
C/C	8	0.08	11	0.11	0.74 (0.28-1.91)
C/T	78	0.78	56	0.54	3.04 (1.65-5.60)
T/T	14	0.14	37	0.35	0.30 (0.15-0.59)
C	94	0.47	78	0.38	1.48 (1.00-2.19)
T	106	0.53	130	0.62	0.67 (0.46-1.00)

Tab. I. The distribution of genotypes, frequency of alleles of the C-460T polymorphism of the VEGF-1 gene and odds ratio (OR) in wet form of age related macular degeneration (AMD) patients and controls without AMD.

Tab. I. Rozkład genotypów i częstość alleli polimorfizmu C-460T genu VEGF-1 oraz analiza ilorazu szans (OR) dla chorych z mokrą postacią zwyrodnienia plamki związanego wiekiem (AMD) i osób bez AMD (kontrola).

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(χ^2=45.12, $p < 0.001$) difference in genotype distributions between patients and controls. An intermediate association (odds ratio 3.04, 95% confidence interval 1.65-5.60) was found between wet form of AMD and the C/T genotype. On the other hand, the T/T genotype displayed the protective effect against wet form of AMD (OR 0.30, 95% CI 0.15-0.59). In general, the C allele increases the risk of AMD in wet form and the T allele has a protective influence against this disease.

Commonly accepted genetic factors indicating occurrence/progression of a disease are mutations, which can contribute to the disease phenotype, especially if they occur with a relatively high frequency, taking form of gene polymorphisms. The progression of AMD from its dry to wet form is still unclear and is a matter of discussion. This justify research on specific indicators, which can be attributed to this disease. Furthermore, the correlation of VEGF production, initiated by different physiological stimuli, with a specific genotype may allow identification of individuals who exhibit high or low levels of VEGF production predisposing them to VEGF-mediated pathologies.

Intense angiogenesis is a hallmark of the wet form of AMD and is underlying by high activity of VEGF. This activity may be a consequence of alternative splice of the *VEGF* gene, which can be spliced to form the angiogenic (VEGFxxx) and potentially anti-angiogenic (VEGFxxxb) families of isoforms (8,9). The regulation of splicing originating from upstream regions of the gene could result in alterations in the recruitment of splicing factors to the RNA polymerase complex and control of the speed of the polymerase reaction. It is suggested that some of the upstream polymorphisms, e.g. G-460C could affect this process. Recently, it was showed that in the eye of diabetic patients VEGF splicing was switched from an antiangiogenic to a pro-angiogenic environment and -460C allele might be critical factor for the balance of VEGF isoforms (10). In our previous work we showed that the C-460T polymorphism of the *VEGF* gene may be associated with diabetic retinopathy when combined with another polymorphism of the promoter of this gene, G-634C (11).

In summary, the C-460T polymorphism of the endothelial growth factor can be considered as a potential marker for the wet form of age-related macular degeneration.

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