

RESEARCH ARTICLE

Stromal Cell-Derived Factor 1 Polymorphism in Retinal Vein Occlusion

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Abstract

Background

Stromal cell-derived factor 1 (SDF1) has crucial role in the regulation of angiogenesis and ocular neovascularisation (NV). The purpose of this study was to evaluate the association between *SDF1-3'G(801)A* polymorphism and NV complications of retinal vein occlusion (RVO).

Methods

130 patients with RVO (median age: 69.0, range 35–93 years; male/female— 58/72; 55 patients had central RVO, 75 patients had branch RVO) were enrolled in this study. In the RVO group, 40 (30.8%) patients were diagnosed with NV complications of RVO and 90 (69.2%) patients without NVs. The median follow up period was 40.3 months (range: 18–57 months). The *SDF1-3'G(801)A* polymorphism was detected by PCR-RFLP. Allelic prevalence was related to reference values obtained in the control group consisted of 125 randomly selected, age and gender matched, unrelated volunteers (median age: 68.0, range 36–95 years; male/female— 53/72). Statistical analysis of the allele and genotype differences between groups (RVO patients vs controls; RVO patients with NV vs RVO patients without NV) was determined by chi-squared test. P value of <0.05 was considered statistically significant.

Results

Hardy-Weinberg criteria was fulfilled in all groups. The *SDF1-3'G(801)A* allele and genotype frequencies of RVO patients were similar to controls (*SDF1-3'A* allele: 22.3% vs 20.8%; *SDF1-3'(801)AA*: 5.4% vs 4.8%, *SDF1-3'(801)GG*: 60.8% vs 63.2%). The frequency of *SDF1-3'(801)AA* and *SDF1-3'(801)GA* genotypes, as well as the *SDF1-3'(801)A* allele frequency were higher in RVO patients with NV versus in patients without NV complication (*SDF1-3'(801)AA+AG* genotypes: 57.5% vs 31.1%, $p = 0.008$; *SDF1-3'(801)A* allele: 35.0% vs 16.7%, $p = 0.002$) or versus controls (*SDF1-3'(801)AA+AG* genotypes 57.5% vs 36.8%, $p = 0.021$; *SDF1-3'(801)A* allele: 35.0% vs 20.8% $p = 0.01$). Carrying of *SDF1-3'(801)A*

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Citation: Szigeti A, Ecsedy M, Schneider M, Lénárt L, Lesch B, Nagy ZZ, et al. (2016) *Stromal Cell-Derived Factor 1* Polymorphism in Retinal Vein Occlusion. PLoS ONE 11(11): e0166544. doi:10.1371/journal.pone.0166544

Editor: Graham R. Wallace, University of Birmingham, UNITED KINGDOM

Received: May 8, 2016

Accepted: October 31, 2016

Published: November 10, 2016

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was funded by grants of OTKA K112629- and MTA-SE Lendület Diabetes Research Grant (LP-008/2015) and BELSZEMMED Kft. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The authors have no financial or proprietary interest in any material or method mentioned.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: RVO, retinal vein occlusion; BRVO, branch retinal vein occlusion; CRVO, central retinal vein occlusion; SDF1, stromal cell-derived factor 1; HSCs, hematopoietic stem cells; EPCs, endothelial progenitor cells; NV, neovascularisation; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; VCAM-1, vascular cell adhesion molecule-1; OR, odds ratio; CI, confidence interval; Bp, base pairs; VEGF, vascular endothelial growth factor; ROP, retinopathy of prematurity.

allele increased the risk of neovascularisation complications of RVO by 2.69 (OR, 95% CI = 1.47–4.93).

Conclusion

These findings suggest that carrying *SDF1*-3'(801)A allele plays a role in the development of neovascular complications in retinal vein occlusion.

Introduction

Retinal vein occlusion (RVO) is the most common form of retinal vascular disease following diabetic retinopathy with a prevalence rate of 5.2/1000, and may result in permanent vision loss. [1–2]

Beside macular edema, the most serious complication of RVO is neovascularisation (NV), causing vitreous haemorrhage, neovascular glaucoma and fibrovascular membranes with consecutive tractional retinal detachment, which further impairs vision. [3–5] The greatest risk of developing anterior segment NV is during the first 7–8 months after the event, then the risk dramatically falls to minimal. [3,6] In a recent review of central retinal vein occlusion (CRVO) cases the NV incidence was around 50% at 6 months after the occlusion where the ischemic subtype was not defined, while in ischemic CRVO the incidences of iris, angle NV and neovascular glaucoma were 57.7%, 47.4% and 33.3%. [5, 7]

Incidence of NV in branch retinal vein occlusion (BRVO) is difficult to estimate, retinal NV develops in 22–31% of the patients with BRVO. [8–10] NVs occur mostly at the border of perfused and non-perfused retina, while NV at the disc and iris is rare. [3–4] The risk of NV increases when the area of capillary nonperfusion exceeds 5 disc diameters. [10]

Stromal cell-derived factor 1 (*SDF1*) also known as CXCL12 is a highly active small (68-amino-acid, 8 kDa) chemokine, which is responsible for chemotaxis of CXCR4 expressing cells including CD34⁺ hematopoietic stem cells (HSCs), monocytes, lymphocytes, and endothelial progenitor cells (EPCs). Through complex interactions with adhesion molecules, *SDF1* promotes transendothelial migration and attachment of CD34⁺ HSCs to the vascular endothelium. [11–13] The existence of a regulatory loop between VEGF-A and *SDF1* supports the crucial role of the *SDF1* in the regulation of angiogenesis and formation of the new blood vessels in the eye. [10, 14] Higher intravitreal *SDF1* protein level has been measured in RVO patients with NV when compared to patients without NV, or negative controls, suggesting a pivotal role of *SDF1* in the development of neovascular changes during RVO. [15]

SDF1-3'G(801)A polymorphism is the most studied single nucleotide polymorphism in *CXCL12* gene which encodes for SDF1 [16]. The *SDF1*-3'G(801)A allele is the possible target of cis-acting factors enhancing the expression of *CXCL12* gene and the stability of the mRNA transcript. Furthermore *SDF1*-3'(801)A allele carrying homozygotes (*SDF1*-3'(801)AA) have higher SDF1 protein level, than *SDF1*-3'(801)A allele carrying heterozygotes (*SDF1*-3'(801)AG) or wild *SDF1*-3'(801)G allele carrying homozygotes (*SDF1*-3'(801)GG). [17–18].

Since the relevance of *SDF1* in retinal neovascularisation has been already highlighted and the *SDF1*-3'G(801)A polymorphism has been shown to increase the production of *SDF1* the purpose of this study was to evaluate the potential association between *SDF1*-3'G(801)A polymorphism and NV complications of RVO.

Patients and Methods

This prospective controlled study was performed at the Department of Ophthalmology and at the Laboratory for Genetics, 1st Department of Pediatrics, Semmelweis University, Budapest, Hungary. All participants were treated in accordance with the tenets of the Declaration of Helsinki. Institutional Review Board approval was obtained for all study protocols (Semmelweis University Regional and Institutional Committee of Sciences and Research Ethics). Written informed consent was obtained from all participants in this study.

Inclusion criteria: Patients with retinal vein occlusion diagnosed at the Retinal and Macular Diseases outpatient unit of the Department of Ophthalmology, Semmelweis University between January 2010 and January 2013 and commitment to at least 18 months' follow-up.

Exclusion criteria: Patients with a history of previous intraocular surgery, eye trauma, any other retinal or neurological disease (e.g. multiple sclerosis), intraocular inflammation or tumor were excluded from the study.

The control group consisted of 125 randomly selected age and gender matched, unrelated volunteers (median age: 68.0 years, range: 36–95 years, male/female– 53/72), who were referred for spectacle prescription, general routine ophthalmic follow-up (because of diabetes mellitus or hypertension) or for driver's license permit renewal. All cases and controls were Caucasians. Inclusion and exclusion criteria were the same for the RVO group and the age and gender matched control group except for the fact that patients in the control group had no RVO.

Clinical data: RVO was classified according to the anatomical location of the occlusion. BRVO was diagnosed as "major" when one of the major branch veins draining one of the retinal quadrants was occluded. "Macular" BRVO diagnosis was used to describe cases when occlusion is limited to a small vessel draining a sector of the macular region. [19]

Slit-lamp biomicroscopy, intraocular pressure measurement with applanation tonometry, indirect ophthalmoscopy following pupil dilation, fundus fluorescein angiography, gonioscopy were used to identify eyes with NV complications of RVO during the follow up period. If the iris neovascularisation was questionable, we performed anterior segment fluorescein angiography. If there was any suspicion for posterior segment neovascularisation, retinal fluorescein angiography was performed. All patients with RVO also underwent systemic examinations, including fasting blood glucose level determination, systemic blood pressure measurement and detailed cardiovascular and hematological examination.

SDF1-3'G(801)A genotyping

Blood samples taken for regular routine checkup were used for genotyping. Genomic DNA was extracted from whole blood by using the QIAamp Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA samples were then stored at -20°C for further use.

The SDF1-3'G(801)A polymorphism was detected with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method by *MspI* (HpaII) restriction enzyme (Sigma Chemical Co., Budapest, Hungary) digestion from the fragment of 302 bp DNA, previously amplified with the following primers: forward: 5' CAGTCAACCTGGGCAAAGCC3' and reverse: 5' AGCTTTGGTCC TGAGAGTCC 3'). The following program was used for fragment amplification: one cycle of 94°C for 5 min, 94°C for 1min (denaturation), 1min at 50°C for annealing, 72°C for 1 min (elongation) followed by 34 cycles of 94°C for 1min, 50°C for 1 min, and 72°C for 1 min. The cleaved PCR products were electrophoresed on 3% agarose gel stained with ethidium bromide. Samples exhibiting only 302-bp band were assigned as SDF1-3'(801) AA genotype, and samples revealing two bands of 202 and 100 bp were typed as SDF1-3'(801)

GG genotype, and samples illustrating three bands of 302, 202, and 100 bp were assigned as SDF1-3'(801)GA genotype. (Fig 1)

Statistical Analysis

Statistical analyses were performed using SPSS software program (Statistical Package for Social Sciences, SPSS version 22.0; SPSS Inc., Chicago, IL, USA).

Differences between demographic data of RVO patients and control group were assessed by Chi-square test for categorical variables (gender, presence of hypertension and diabetes mellitus). The distribution of the age of patients with RVO and control patients were tested by Kolmogorov-Smirnov and Shapiro-Wilk tests and since the data was not normally distributed we chose Mann-Whitney U nonparametric test for the comparison of the age in different groups. Allele and genotype frequencies were calculated in patients and healthy controls by direct counting.

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed by Arlequin software (version 3.5.2.2; Computational and Molecular Population Genetics Lab, Bern, Switzerland) for both the polymorphisms in RVO patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis.

Statistical analysis of the allele and genotype differences between groups was determined by chi-squared test, and if the expected frequencies were 5 or less, we used Fisher's exact probability test. Risks were examined by odds ratios (OR) and 95% confidence intervals (95% CI) were calculated with the corresponding Chi-square distribution test. P values <0.05 were considered as significant.

Results

Clinical data

After the exclusion of subjects with less than 18 months of follow-up we had 130 patients with RVO (median age: 69.0 years, range: 35–93 years, male/female– 58/72) in this study. Fifty-five

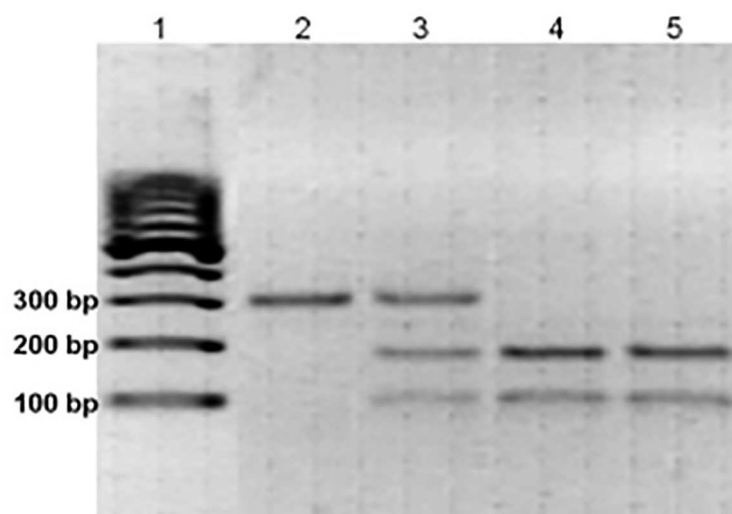


Fig 1. Representative sample of the genotyping for SDF1-3'G(801)A by PCR-RFLP. *Msp I* digestion of the PCR product of *SDF1* gene. DNA molecular weight marker is shown in lane 1. Lane 2 represents sample at 302 bp denoting SDF1-3'homozygous (801)AA mutant genotype, lane 3 shows bands at 302, 202 and 100 bp denoting heterozygous mutant SDF1-3'(801)GA and lanes 4 and 5 at 202 and 100 bp denoting the wild type SDF1-3'(801)GG genotype.

doi:10.1371/journal.pone.0166544.g001

(42.3%) patients had CRVO, 75 (57.7%) patients had BRVO. In the BRVO group 54 patients (72.0%) had major BRVO and 21 patients (28.0%) had macular BRVO. Fifty-three patients (70.7%) had superotemporal BRVO and 22 (29.3%) had inferotemporal BRVO in the BRVO group. Right eye was affected in 63 patients (48.5%) and left eye in 67 patients (51.5%). The mean follow-up of RVO patients was 40.3 months (range: 18–57 months). During the follow up period, 40 (30.8%) patients (24 in CRVO group, 16 in major BRVO group) developed NV in the RVO group. The 24 patients with CRVO developed anterior segment NV, 7 of them were diagnosed with neovascular glaucoma, 16 of the BRVO patients were diagnosed with retinal NV.

The characteristics of the patients and controls are summarized in Table 1. No significant differences were found between the RVO patients and the control group and RVO patients with and without NV in main clinical parameters including age, gender and main general risk factors (diabetes mellitus, hypertension).

Genotype frequencies and allele distribution

Genotype frequencies fulfilled the Hardy-Weinberg equilibrium criteria in all groups ($p = 0.967$ for all participants, $p = 0.930$ for controls, $p = 1.0$ for RVO patients).

The *SDF1* 3’G(801)A allele and genotype frequencies of RVO patients were similar to controls. ($p > 0.05$, see Table 2.)

In the group of RVO patients diagnosed with NV complications, both the frequency of the *SDF1*-3’(801)A allele, as well as the *SDF1*-3’(801)AA genotype were higher than in RVO patients without NV complications. *SDF1*-3’(801)A allele (35.0% vs 16.7%, $p = 0.002$) and *SDF1*-3’(801)AA genotype (12.5% vs 2.2%, $p = 0.028$) increased the risk of NV complications (A allele: 2.69 [OR; 95% CI = 1.47–4.93]; AA genotype 6.29 [OR; 95% CI = 1.17–33.93])

The frequency of *SDF1*-3’(801)AA and *SDF1*-3’(801)AG genotypes in RVO group with NV was higher than in the group of RVO patients without NV complications too. (57.5% vs 31.1%, $p = 0.008$) *SDF1*-3’(801)AA and (801)AG genotype was found to increase the risk of NV complications by 3.00 [OR; 95% CI = 1.39–6.47]

Neither the allele nor the genotype frequencies were different in RVO patients without NV when compared to controls (*SDF1*-3’(801)A allele: 16.7% vs 20.8%, $p = 0.282$; *SDF1*-3’(801)AA genotypes: 2.2% vs 4.8% $p = 0.473$; *SDF1*-3’(801)AA+AG genotypes 31.1% vs 36.8%, $p = 0.386$). However in RVO patients diagnosed with NV complications, both the frequency of

Table 1. Patients and controls characteristics.

Variables	RVO patients	Control patients	P values
Number	130 (55 CRVO, 75 BRVO)	125	
Gender (male/female)	58/72	53/72	0.818 [†]
Age (median, range, years)	69.0 (35–93)	68.0 (36–95)	0.712 ^{††}
Hypertension (n, %)	70 (53.8%)	66 (52.8%)	0.967 [†]
Diabetes mellitus (n, %)	25 (19.2%)	31 (24.8%)	0.356 [†]
Variables	RVO patients without NV	RVO patients with NV	P values
Number	90 (31 CRVO, 59 BRVO)	40 (24 CRVO, 16 BRVO)	
Gender (male/female)	38/52	20/20	0.527 [†]
Age (median, range, years)	67.5 (35–90)	70.0 (37–93)	0.226 ^{††}
Hypertension (n, %)	43 (47.8%)	27 (67.5%)	0.059 [†]
Diabetes mellitus (n, %)	14 (15.6%)	11 (27.5%)	0.176 [†]

Chi-squared test for categorical variables (†) and Mann-Whitney U test for continuous variable (††).

doi:10.1371/journal.pone.0166544.t001

Table 2. *SDF1*-3'G(801)A allele and genotype frequencies in control and RVO group and p values of significance regarding the comparison between the RVO patients and the controls.

Alleles	A allele (n, %)	G allele (n, %)	P-value
RVO patients	58 (22.3%)	202 (77.7%)	
Control patients	52 (20.8%)	198 (79.2%)	0.759
Genotypes	AA	AG+GG	P-value
RVO patients	7 (5.4%)	123 (94.6%)	
Control patients	6 (4.8%)	119 (95.2%)	0.832
Genotypes	AA+AG	GG	P-value
RVO patients	51 (39.2%)	79 (60.8%)	
Control patients	46 (36.8%)	79 (63.2%)	0.787

SDF1-3'G(801)A allele and genotype frequencies in RVO patients with (n = 40) or without NV (n = 90) are summarized in Table 3.

doi:10.1371/journal.pone.0166544.t002

the *SDF1*-3'(801)A allele and *SDF1*-3'(801)A carrying genotypes (*SDF1*-3'(801)A allele: 35.0% vs 20.8%, p = 0.01; *SDF1*-3'(801)AA+AG genotypes 57.5% vs 36.8%, p = 0.021) was higher than in control group.

Discussion

According to our knowledge this is the first investigation focusing on the relevance of *SDF1*-3'G(801)A polymorphism in patients with retinal vein occlusion. We found significant association between the development of neovascular complications and the presence of the *SDF1*-3'G(801)A allele.

Neovascular complications usually occur in the first 7–8 months in CRVO and 6–12 months in BRVO [3–7] In order to minimize the early drop-out of patients we only enrolled those participants whose follow-up was ensured for at least 18 months.

In this present study the frequency of the *SDF1*-3'(801)A allele in RVO patients with NV complications was higher than in RVO patients without NV complications resulting in an almost tripled risk increase. The frequencies of homozygous *SDF1*-3'(801)AA and *SDF1*-3'(801)GA heterozygous genotypes were also higher in RVO patients with NV complications than in patients without NV and controls. These findings suggest that carrying of *SDF1*-3'(801)A allele predisposes for the development of NV complications in patients with RVO.

Table 3. *SDF1*-3'G(801)A allele and genotype frequencies in RVO patients with (n = 40) or without NV (n = 90) and p values of significance regarding the comparison between the two groups of RVO patients.

Alleles	A allele (n, %)	G allele (n, %)	P	OR (95% CI)
RVO patients with NV	28 (35.0%)	52 (65.0%)	0.002	2.69 (1.47–4.93)
RVO patients without NV	30 (16.7%)	150 (83.3%)		
Genotypes	AA	AG+GG	P	OR (95% CI)
RVO patients with NV	5 (12.5%)	35 (87.5%)	0.028[‡]	6.29 (1.17–33.93)
RVO patients without NV	2 (2.2%)	88 (97.8%)		
Genotypes	AA+AG	GG	P	OR (95% CI)
RVO patients with NV	23 (57.5%)	17 (42.5%)	0.008	3.00 (1.39–6.47)
RVO patients without NV	28 (31.1%)	62 (68.9%)		

[‡]Fisher's exact test

doi:10.1371/journal.pone.0166544.t003

SDF1 is essential for the migration, homing and differentiation of circulating HSCs and EPCs during the retinal neovascularisation process. [11–12, 20–21] In experimental models, *SDF1* was found to be expressed and upregulated in an ischemia model of the retina in rats. [22–23] *SDF1* induced CXCR-4 (seven-transmembrane domain G-protein coupled receptor) activation leads to β -integrin expression and the binding of vascular cell adhesion molecule (VCAM)-1 to endothelial cells. [24–25] In retinal endothelial cells increased *SDF1* expression resulted in a higher VCAM-1 production [12], which plays an important role in HSCs homing and mobilization. [12, 26] It has been also demonstrated that *SDF1* had an effect on retinal endothelial cells and on the occludin gap junction protein, which is responsible for the tight junctions between endothelial cells to prevent leakage of the vessel. The retinal endothelial expression of occludin is decreased with increasing *SDF1* level. [11–12]

Only a few previous studies have investigated the vitreous level of *SDF1* in ocular diseases with neovascularisation complications. [15, 27–29] Chen et al found higher *SDF1* levels in patients with proliferative diabetic retinopathy than in non-diabetic controls. Furthermore in proliferative diabetic retinopathy patients the vitreous concentration of *SDF1* correlated well with the VEGF level. [27] In another study using a murine model of proliferative adult retinopathy exogenous *SDF1* promoted neovascularization further [11–12]. Vitreous *SDF1* alpha level was also increased in eyes with vascularly active stage 4 retinopathy of prematurity (ROP) versus either inactive stage 4 ROP or control eyes. [29]

In RVO patients higher intravitreal *SDF1* levels were measured in the presence of NV complications compared to RVO patients without NV complications or controls. [15]

All these data suggest that *SDF1* is a prominent contributor in the progression of retinal neovascularisation with a special emphasis on the angiogenic changes during RVO. [15] Higher VCAM-1 production and decreased occludin levels may be involved in the breakdown of the blood-retinal barrier in RVO. *SDF1* could promote neovascularisation by causing a disruption of the blood-retinal barrier through interactions with VCAM-1, β -integrin and occludin.

SDF1 gene (also known as CXCL12 gene) is located on human chromosome 10q11.1. In the single nucleotide polymorphism of *SDF1*-3'G(801)A is mutated to A at position 801 in 30-untranslated region in the b transcriptional splice variant. [16–17]

The allelic frequency of *SDF1*-3'G(801)A was reported to vary between 0.16–0.21 in Caucasian population which is in line with the prevalence of *SDF1*-3'(801)AA and AG genotypes in our study population. [30–33] Gu XL et al. [18] recently described that both mRNA expression and protein level of *SDF1* is increased in the monocytes of healthy subjects with *SDF1*-3'(801)AA than those with GA or GG genotypes.

Djuric Z et al. found that the *SDF1*-3'(801)AA genotype is more frequent in patients with proliferative diabetic retinopathy and pointed to a possible role of this allelic variant in the development of proliferative diabetic retinopathy. [30]

The incidence of diabetes mellitus in our RVO patients was 19.2%, in previous studies it ranged from 14% to 34% [6, 34] According to a previous study by Dubois-Laforgue et al. [33] *SDF1*-3'(801)A polymorphism was similar in Caucasian patients with or without diabetes mellitus.

The effect of diabetes mellitus on the risk of developing NV in RVO is controversial. Previous studies found higher rates of NV in diabetic patients with CRVO than in non-diabetic patients with CRVO. [35–36] Others reported no significant association between diabetes and neovascular complications in retinal vein occlusion. [7]

We did not exclude diabetic patients from our study only those patients who had diabetic retinopathy that could mimic RVO and induce neovascular proliferation. We did not find significant difference in the presence of diabetes mellitus among the RVO patients with and

without NV in our study. In order to include the presence of diabetes mellitus as possible confounding systemic risk factor in the statistical analysis, a larger sample size would be needed. This is a limitation of our study.

None of our BRVO patients did develop neovascular glaucoma. It is in concordance with observation published by Hayreh et al. They found that in BRVO the retina and optic disc were the major sites of NV and none of their BRVO eyes (191 major BRVO, 74 macular BRVO eyes) did develop neovascular glaucoma. [3]

None of our patients with CRVO did develop retinal NV. This finding is in concordance with the observation published by Hayreh et al. that in CRVO the chance for the development of anterior segment NV is much greater than for posterior segment NV. [7] In this study, we investigated the association between SDF1-3'G(801)A polymorphism and presence of SDF1-3'G(801)A allele and NV complications in patients with retinal vein occlusion for the first time. Due to our relatively small sample size, we did not evaluate the polymorphism in subgroups of different types of RVO. Our preliminary results should be confirmed in a larger cohort with subgroup analysis.

There are certain limitations to our study. First, it should be noted that all of our study participants were Hungarian white Caucasians, and thus, the possibility of ethnicity as a confining factor could be excluded. Second, here we evaluated hypertension and diabetes mellitus as possible risk factors. Other systemic risk factors such as hyperlipidemia, cardiovascular diseases, blood hyperviscosity and diseases with hypercoagulation were not included. Further data collection is in progress to analyze these values as possible limiting factors. Third, due to our relatively small sample size, these are rather preliminary results which should be confirmed in larger cohorts and in subgroups of different types of RVO as well.

Conclusion

In summary, this is the first study evaluating *SDF1-3'G(801)A* polymorphism in patients with retinal vein occlusion. We found significantly higher frequencies of the *SDF1-3'(801)A* allele and *SDF1-3'(801)AA* and *SDF1-3'(801)GA* genotypes in RVO patients with NV compared to patients without NV and to controls. Since *SDF1-3'G(801)A* polymorphism has been shown to increase the production of *SDF1* protein, which could promote neovascularisation by causing a disruption of the blood-retinal barrier, presence of *SDF1-3'(801)A* allele may be genetic risk factors for NV complications after RVO.

Supporting Information

S1 Table. Demographical characteristics (age, gender, main general risk factors: diabetes mellitus, hypertension, follow-up time, type of RVO) and *SDF-1 3'G(801)A* polymorphism data of RVO patients.

(XLS)

S2 Table. Demographical characteristics (age, gender, presence of diabetes mellitus, hypertension) and *SDF-1 3'G(801)A* polymorphism data of our study control subjects.

(XLS)

Acknowledgments

This study was founded by grants of OTKA K112629- and MTA-SE Lendület Diabetes Research Grant (LP-008/2015) and BELSZEMMED Kft.

The authors have no financial or proprietary interest in any material or method mentioned.

We thank Mrs. Zoltánné Gizella Ancsin and Mrs. Lászlóné Éva Varga for coordinating our study participants and Ms Mária Bernáth for helping in genotype analysis. We thank Janos Mulvai for helping in editing of [Fig 1](#).

Author Contributions

Conceptualization: AS ZR AF.

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Formal analysis: MS AS BL LL ZR.

Funding acquisition: AS MS AF ZZN ZR.

Investigation: AS MS ME BL LL ZR ZZN.

Methodology: AS ZR AF.

Project administration: AS ME MS BL ZR.

Resources: ZR AF ZZN.

Software: LL AF.

Supervision: AS ME MS BL AF ZZN ZR.

Validation: AS LL AF.

Visualization: AS ME MS BL AF ZZN ZR.

Writing – original draft: AS ME MS BL AF ZZN ZR.

Writing – review & editing: AS ME MS BL AF ZZN ZR.

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