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Analysis of functional polymorphisms in apoptosis-related genes in primary open angle glaucoma

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Purpose: Glaucoma is a disease with high heritability in which the degradation of retinal ganglion cells occurs via apoptosis. Therefore, we investigated the role of four functional apoptosis-related gene variants (Akt1 rs1130233, Bax rs4645878, Fas rs223476, and FasL rs763110) in patients with primary open angle glaucoma.
Methods: 334 patients with primary open angle glaucoma and 334 controls were recruited for this case–control study. The main outcome measures were genotype distribution and allelic frequencies determined with PCR.
Results: After adjustment for multiple testing, no significant difference in either the genotype distribution or the allelic frequencies of any investigated gene variant was found.
Conclusions: Our findings indicate that the investigated gene polymorphisms are unlikely to be major risk factors for primary open angle glaucoma in Caucasian patients.

Glaucoma is defined as a group of progressive optic neuropathies characterized by a steady loss of retinal ganglion cells and consequently visual field defects. The number of people with glaucoma is about 60 million worldwide and will exceed 110 million by 2040 [1]. The pathomechanisms that lead to retinal ganglion cell (RGC) death are not fully understood. High intraocular pressure (IOP) is the main risk factor, but in the case of normal tension glaucoma, glaucomatous nerve degeneration occurs although the IOP is within normal limits.

Thus, other factors, such as ocular blood flow [2], inflammation [3], and genetics, must be considered. A positive family history for glaucoma is known to increase individual risk substantially [4]. There is no clear Mendelian inheritance pattern, but some portion of glaucoma is caused by defects in distinct genes, for example, myocilin [5]. The genetics of the larger portion of glaucoma is more complex, and a vast number of gene variants have been implicated [6].

Apoptosis or programmed cell death is a complex process, which ultimately leads to cellular degradation [7]. In glaucoma, several studies emphasized the role of apoptotic factors, such as apoptosis stimulating fragment (FAS) [8], BCL2-associated X protein (BAX) [9], and v-akt murine thymoma viral oncogene homolog 1 (Akt1) [10]. The binding of FAS ligand (FASL) to FAS forms the death-inducing signaling complex, which triggers apoptosis. FAS ligand (FASL) deficiency protects RGCs from cell death [10]. AKT is a serine-threonine protein kinase, which affects the components of the intrinsic cell death machinery [11,12]. The BAX protein is a member of the BCL2 family and has proapoptotic properties by antagonizing BCL2 [13]. Recently, C*HSDGIC*, a novel cyclopeptide from the cyclization of pituitary adenylate cyclase-activating polypeptide, was shown to inhibit apoptosis in retinal ganglion cells by decreasing BAX and increasing BCL2 [14].

For this candidate gene analysis, we selected polymorphisms that had already been demonstrated to alter gene expression or the activity of apoptosis-related genes [15-18]. Identifying the associations of gene polymorphisms with primary open angle glaucoma (POAG) may enhance our understanding of the pathogenesis and may pave the way for future neuroprotective therapies. In addition, a more precise individual risk profile can provide assistance for screening and foreseeing the progression rate of the condition. Therefore, we investigated a hypothesized association between apoptosis-related functional gene polymorphisms and POAG.

METHODS

In the present institutional, retrospective case-control study we investigated a total of 668 unrelated Caucasian subjects comprising 334 patients with POAG, and 334 control subjects. In each group 187 females and 147 males were involved. The mean age of patients with POAG was 74.1 ± 8.1 years and 73.2 ± 7.8 years in control subjects, respectively. All participants were of Caucasian origin, living in the same geographical area and were seen at the local Department of Ophthalmology, Medical University of Graz. The study was approved by the Institutional Review Board of the Medical

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Table 1. Primer nucleotide sequences.

Gene	SNP	Primer (5'-3')
AKT1	rs1130233	F: AGCTGTTCTTCCACCTGTML
		R: TCTCCGAGTGCAGGTAGTML
BAX	rs4645878	F: ACCCTGCCCGAAACTTCTAA
		R: GAGCATCTCCCGATAAGTGC
FAS	rs2234767	F: CCTTATCCCACTTCTTTTTGTGTC
		R: GGCTTGTCTCTGTTCCACCT
FASL	rs763110	F: CTGGGCAAACAATGAAAATG
		R: ACCCACTTTAGAAATTAGATCA

University of Graz and followed the principles of the Declaration of Helsinki. The study adhered to the ARVO statement on human subjects. Prior to enrollment, written informed consent was obtained from all participants.

All patients underwent slit-lamp biomicroscopy, testing for best-corrected visual acuity, Goldmann applanation tonometry, gonioscopy, and standard automated perimetry (Interzeag Octopus 500, program G2) or, in cases of profoundly decreased visual acuity, Goldmann perimetry. In all patients, photographs of the optic discs were taken.

POAG was defined by the IOP before the initiation of a pressure-lowering therapy of at least 21 mmHg, an open anterior chamber angle, optic disc changes characteristic of glaucoma (notching, thinning of the neuroretinal rim, increased cup/disc ratio in relation to the optic disc size), visual field defects characteristic of glaucoma (inferior or superior arcuate scotoma, nasal step, paracentral scotoma), and the absence of conditions that lead to secondary glaucoma. A narrow angle was defined as Schaffer's grade 2 or less and was excluded with gonioscopy.

The control group consisted of 334 unrelated patients with no morphological or functional damage indicative of primary or secondary open angle or angle closure glaucoma. The control subjects had been admitted to the department for cataract surgery. All participants were Caucasians from the same geographic area (southern Austria).

Genotype determination: Venous blood was collected in 5 ml EDTA tubes. DNA was isolated form peripheral lymphocytes using a QIAamp DNA blood mini-kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol and stored at −20 °C. Genotype determination was performed using high-resolution melting curve analysis on the LightCycler® 480 PCR system (Hoffmann-La Roche, Basel, Switzerland). The samples were amplified in duplicate 20 µl reactions using the Light Cycler 480 High Resolution Melting Master kit (Roche Diagnostics, Wien, Austria) and analyzed on an LC480 instrument I (Roche Diagnostics GmbH, Mannheim, Germany). The final reaction mix contained 1X Master Mix, 3 mM $MgCl_2$, 4 µM forward and reverse primer, and 50 ng of genomic DNA. Primer sequences are shown in Table 1. For PCR, the following cycling conditions were chosen: one cycle of 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. The amplicons were then denaturated at 95 °C for 1 min, cooled down to 40 °C for 1 min, and then melted from 65 °C to 95 °C with 25 signal acquisitions per degree. To detect sequence variations, Gene Scanning Software v 1.5 (Roche Diagnostics) was used. Using the Auto Group, mode samples were automatically grouped because of their melting curves.

Statistical analysis: Descriptive statistics were used to calculate the frequencies and percentages of discrete variables. Continuous data are given as mean ± standard deviation (SD). Means were compared using the Mann–Whitney test. Proportions of groups were compared with a chi-square test. The criterion for statistical significance was p value less than 0.05. The Bonferroni correction was used to adjust for multiple testing. Hardy–Weinberg equilibrium was calculated using HW Diagnostics-Version 1.beta (Fox Chase Cancer Center). Statistical analysis was performed using the SPSS statistical package (SPSS, version 17.0, Chicago, IL). Power calculation was done using PS Power and Sample Size Calculation software version 2.1.30.

RESULTS

Our study included 334 patients with POAG and an equal number of control subjects. In each group, 187 women and 147 men were included. The mean age of the patients with POAG and the control subjects was 74.1±8.1 years and 73.2±7.8 years, respectively.

The observed genotype distributions did not deviate from those predicted by Hardy–Weinberg equilibrium. Table 2 shows the genotype and minor allele frequencies of the

Table 2. Distribution of the investigated gene polymorphisms.

Gene	Mutation	POAG	Controls	P-value	Odds ratio
Akt1	G/G	216	206	0.422	
rs1130233	A/G	99	116	0.159	
MAF	A/A	19	8	0.031	0.80 (0.62–1.05)
	A	0.21	0.2	0.81	
Bax	G/G	259	254	0.647	
rs4645878	A/G	66	75	0.393	
MAF	A/A	9	5	0.28	1.01 (0.73–1.40)
	A	0.13	0.13	0.87	
Fas	G/G	275	265	0.326	
rs2234767	A/G	54	65	0.266	
MAF	A/A	5	4	0.737	1.16 (0.81–1.65)
	A	0.1	0.11	0.36	
FasL	G/G	132	150	0.159	
rs763110	A/G	159	145	0.277	
MAF	A/A	43	39	0.637	0.87 (0.70–1.08)
	A	0.37	0.33	0.27	

Absolute numbers of patients and controls are shown. Rs-numbers of the investigated polymorphism are depicted below the gene name. Minor allele frequencies (MAF) are indicated in the bottom line. Odds ratios are given with 95% confidence interval in parentheses.

investigated polymorphisms in the patients with POAG and the control subjects. Genotype AA of AKT1 rs1130233 was significantly more common in POAG, but this significance did not remain after the Bonferroni adjustment. Therefore, no significant difference in either the genotype or allelic frequencies of any investigated gene variant was found. Quantitative trait analysis with age at presentation, mean defect in visual fields, vertical cup/disc ratio, or intraocular pressure did not reveal any association ($p>0.05$).

The present study had a statistical power of 0.80 to detect an odds ratio of 1.86 at a significance level of 0.05.

DISCUSSION

To the best of our knowledge, the present study is the first to investigate a hypothesized association between POAG and functional apoptosis-related gene polymorphisms. Genotypes of four functional apoptosis-related polymorphisms were analyzed in 334 patients with POAG and 334 controls matched for age and sex. After correction for multiple testing, the allelic frequencies and genotype distribution did not differ significantly between the groups.

AKT inhibits apoptosis by phosphorylation and, thus, inhibition of transcription factors of the forkhead family, which trigger programmed cell death by inducing FASL [19]. Furthermore, AKT1 influences nerve cell plasticity and development [20]. The influence of rs1130233 on the expression of AKT1 has been demonstrated in lymphoblasts [21]. Although gene variants of AKT1 have been shown to affect apoptosis in a cell model [18], we could not find an association with POAG.

Gene polymorphisms have been shown to affect nuclear protein binding affinity and thus the transcription of BAX [22]. Mice deficient in BAX were protected from retinal ganglion cell death suggesting a major role of BAX in glaucoma development [23]. However, we did not find an association of the BAX gene polymorphism rs4645878 with POAG, which is in line with the findings of a recent meta-analysis in chronic lymphatic leukemia [24]. Despite the functionality of this polymorphism, it was not shown to alter the predisposition of POAG or chronic lymphatic leukemia, two diseases in which apoptosis plays a pivotal role.

Binding of FAS to FASL leads to caspase 8 activation and is a key mechanism of normal tissue homeostasis [25]. Polymorphisms in FAS and FASL have been found to be associated with various inflammatory diseases [26-28] and cancer [29-31]. Moreover, the expression of FAS and FASL is altered in neurodegenerative disorders. The FAS/FASL pathway has already been demonstrated to play a critical role in mediating beta-amyloid-induced death of neurons in

Alzheimer's disease [32,33]. Interestingly, in a study that used enzyme-linked immunosorbent assay (ELISA), FASL levels were not significantly altered in patients with POAG [34], and with multiplex beads analysis, the FASL levels were below the limits of detection in aqueous humor [35]. However, other studies identified the upregulation of FAS/FASL in glaucoma [10,36,37]. Due to these conflicting results, the role of FAS/FASL in glaucoma remains elusive. Still, our findings do not support the pivotal influence of FAS/FASL on glaucoma pathogenesis.

The following potential limitations should be kept in mind when interpreting our results. First, only a small number of single nucleotide polymorphisms (SNPs) were investigated in the present study. We cannot rule out associations with other polymorphisms of the investigated genes. A tagged SNP approach would be a possible strategy here, but it was beyond the scope of this survey. All investigated SNPs had already been demonstrated to be functional. Second, as genetic polymorphisms have been shown to vary between populations, our findings do not necessarily apply to populations other than Caucasian. In conclusion, our findings suggest that the investigated gene polymorphisms are unlikely to be major risk factors for POAG in Caucasian patients.

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