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Association of *MMP1*-1607 1G/2G and *TIMP1* 372 T/C gene polymorphisms with risk of primary open angle glaucoma in a Polish population

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Primary open angle glaucoma (POAG) is considered to be a leading cause of irreversible blindness worldwide. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) have been extensively studied as POAG risk factors. Recently, several single-nucleotide polymorphisms (SNPs) for *MMPs* and TIMPs encoding genes have been reported in POAG patients. The aim of this study was to investigate association of the -1607 1G/2G *MMP1* and 372 T/C *TIMP1* gene polymorphisms with risk of POAG in a Polish population.

Material/Methods: In the present case-control study we examined a group of 449 unrelated Caucasian subjects consisting of 196 POAG patients (66 males and 130 females; mean age 70±14) and 253 controls (72 males and 181 females; mean age 67±16). The *MMP1*-1607 1G/2G and *TIMP1* 372 T/C gene polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The odds ratios (ORs) and 95% confidence intervals (CIs) for each genotype and allele were calculated.

Results: We found a statistically significant increase of the 2G/2G genotype (OR 1.73; 95% CI 1.05–2.86; p=0.019) as well as the 2G allele frequency (OR 1.34; 95% CI 1.03–1.75; p=0.017) of *MMP1* in POAG patients in comparison to healthy controls. There were no differences in the genotype and allele distributions and odds ratios of the *TIMP1* polymorphism between patients and controls group. We also did not find any association of *TIMP1* with *MMP1* gene-gene interaction and risk of POAG occurrence.

Conclusions: In conclusion, we suggest that the -1607 1G/2G polymorphism of *MMP1* gene may be considered as an important risk factor associated with primary open angle glaucoma in a Polish population. However, further *in vivo* study is needed to evaluate biological importance of *MMP*s polymorphisms as a risk factor of POAG.

key words: **open angle glaucoma • MMPs • TIMPs • gene polymorphisms**

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Background

Primary open angle glaucoma (POAG) is one of the main cause of irreversible blindness worldwide [1]. It is characterized by progressive loss of retinal ganglion cells (RGCs) and their axons, leading to the pathognomonic cupping of the optic nerve head [2]. Recent epidemiological studies indicated that apart from high intraocular pressure (IOP) and age, ethnic origin, diabetes mellitus, and genetic factors might be associated with a risk of POAG [3–5]. Mutations in the myocilin and optineurin gene (*MYOC* and *OPTN*) are the most prominent [6,7], but were not found in most patients with POAG [8]. Several single nucleotide polymorphisms (SNPs) in metalloproteinases (MMPs) and tissue inhibitors for metalloproteinases (TIMPs) encoding genes have recently been identified in POAG patients [9–11].

The matrix metalloproteinases constitute a group of neutral, Ca- and Zn-activated endoproteinases involved not only in physiological extracellular matrix (ECM) turnover during embryogenesis and angiogenesis but also in interactions between cells and their surroundings [6,12]. Most importantly, MMPs are responsible for regulating aqueous humor from the anterior chamber of the eye and therefore have a significant impact on intraocular pressure [13,14]. A significantly altered level of MMPs in the aqueous humor of patients with diagnosed POAG has been previously described [13,15].

In order to estimate an association of MMPs with primary open angle glaucoma, we investigated the *MMP1*-1607 1G/2G and the *TIMP1* 372 T/C gene polymorphisms in a Polish population.

Material and Methods

Patients

In the present case-control study we investigated a total of 449 unrelated Caucasian subjects from a Polish population (Table 1). The study was conducted in accordance with the standards of the local ethics committee. The study group consisted of 196 unrelated patients with diagnosed POAG (66 males and 130 females; mean age 70±14) and the control group of 253 unrelated patients without glaucoma symptoms (72 males and 181 females; mean age 67±16). All patients and controls were matched on age (no differences were calculated, P=0.054). All subjects underwent ophthalmic examination, including best-corrected visual acuity, intraocular pressure, slit-lamp examination, gonioscopy, and fundus examination using non-contact and contact fundus lenses with a slit lamp. In the group of glaucomatous patients, the diagnosis of POAG was stated prior to enrolment, in accordance with the guidelines of European Glaucoma Society (*Terminology and Guidelines for Glaucoma, Second Edition, Dogma, Savona 2003, Italy*). The patients with POAG at the time of enrolment in the study were treated topically with 1 or a combination of typical anti-glaucoma medications including beta blockers (Timolol), prostaglandin analogs (Latanoprost), carbonic anhydrase inhibitors (Dorzolamide) and alpha2 agonists (Brimonidine).

Medical history was obtained from all subjects, and none reported present or past cancer or any genetic disease. Patients were excluded from the study if they were subject to any of the following conditions: use of any prescribed eye drops other than anti-glaucoma preparations and artificial tears, any ocular surgeries or laser treatments performed in the past 6 months, and present or past treatment with glucocorticosteroids or immunosuppressive therapy (if these treatments had not been stopped at least 1 year before collection of specimens). All subjects included in the study resided in Warsaw District, Poland. All patients were recruited from the Department of Ophthalmology, Medical University of Warsaw. The study was reviewed and approved by the local ethics committee and met the tenets of the Declaration of Helsinki. Written consent was obtained from each patient before enrolment in the study.

Genotype determination

Blood samples were collected in 3 ml EDTA tubes. DNA was extracted from peripheral lymphocytes using the nucleic isolation kit QIAamp DNA Mini and Blood Kit (Qiagen, Chatsworth, CA, USA) following the manufactures protocol and stored at –20°C. The *MMP1* and *TIMP1* genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to previously described procedures with some modifications (Table 2) [16–19]. Briefly, each 20 µl of the PCR reaction contained 10 ng genomic DNA, 1.25 U Taq polymerase (Qiagen, Chatsworth, CA, USA) in 1×PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, 0.1% gelatine), 1.5 mM $MgCl_2$, 50 mM dNTPs, and 250 nM each primer. Thermal cycling conditions with primer sequences (Sigma-Aldrich, St. Louis, MO, USA) are displayed in Table 1. The PCR was carried out in a MJ Research, INC thermal cycler; model PTC-100 (Waltham, MA, USA).

Primer sequences used in amplification of the *MMP1*-1607 1G/2G and the *TIMP1* 372 T/C gene polymorphic sites are displayed in Table 2. Two mismatches were introduced into the reverse annealing primer of the *MMP1*-1607 1G/2G polymorphism [19], resulting in the restriction endonuclease X*mn*I recognition sequence (5'-GAANNNNTTC-3') for the 1G allele. The *MMP1* PCR amplification product (118 bp) was digested with 1 unit of *Xmn*I (New England Biolabs, Ipswich, MA, USA) for 16 hours and, only in the presence of the 1G allele, it was cut into 2 fragments of 89 bp and 29 bp (Figure 1). The *TIMP1* PCR amplification product (175 bp) was digested with 1 unit *Bss*SI (New England Biolabs, Ipswich, MA, USA) for 16 hours and, only the presence of C allele, it was cut into 2 fragments of 155 bp and 20 bp (Figure 2). The PCR products were separated by 8% polyacrylamide gel electrophoresis.

Data analysis

The allele frequencies were estimated by gene counting and genotypes were scored. The $\chi 2$ test was used to compare the observed numbers of genotypes with those expected for a population in the Hardy-Weinberg equilibrium and to test the significance of the differences of observed alleles and genotypes between groups. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. When calculating the probability, Pearson correction was used, and if the expected cell values were less than 5, Fisher's exact test was used. A P-value of <0.05 was taken as statistically significant. The t-test (for normal distribution) or Mann-Whitney test (for non-normal distribution) was used to compare each

Table.1. The characteristic of open-angle glaucoma (POAG) patients.

Patients	Age (years)	POAG diagnosis (years)	Intraocular pressure, IOP (mmHg)	Best corrected visual acuity, BCVA
Mean	70±14	10.5±5.6	13.1±1.8	0.7±0.16
	Gender male/female	**Hypertension***	**Low blood pressure****	**POAG in family relatives**
Number	66/130	81	44	47

* Systolic pressure ≥140; Diastolic pressure ≥90 mmHg; ** Systolic pressure <90; Diastolic pressure <60 mmHg.

Table.2. Primer sequences and restriction endonucleases used in the *MMP1*-1607 1G/2G and the *TIMP1* 372 T/C gene polymorphisms analysis by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Primer	Sequence	Annealing	Enzyme	Product
MMP1 -1607 1G/2G Forward MMP1 -1607 1G/2G Reverse	5'-TCG TGA GAA TGT CTT CCC ATT-3' 5'-TCT TGG ATT GAT TTG AGA TAA GTG AAA TC-3'	56°C	*XmnI*	118 bp
TIMP1 372 T/C Forward *TIMP1* 372 T/C Reverse	F: 5'-GCACATCACTACCTGCAGTC-3' R: 5'-GAAACAAGCCCACGATTTAG-3'	55°C	*Bss*SI	175 bp

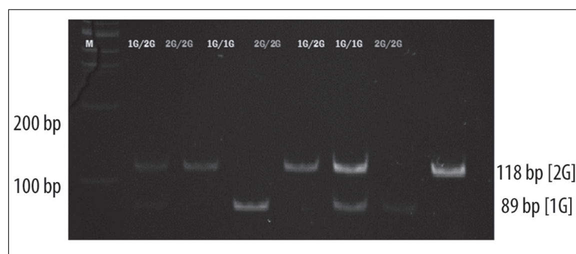

Figure 1. The representative electrophoresis of PCR products of the -1607 1G/2G polymorphic region of *MMP1* gene separated by 8% polyacrylamide gel. Lines: M - DNA marker (100 bp); 3, 6 – homozygote 2G/2G; 1, 5 – heterozygote 1G2G; 2, 4, 7 – homozygote 2G2G.

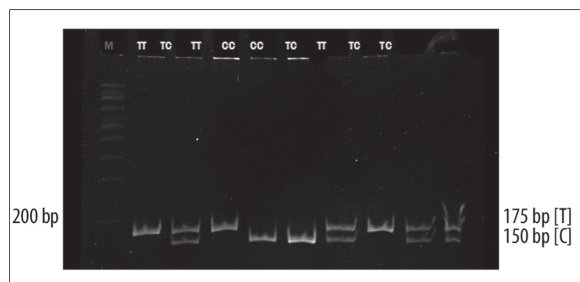

Figure 2. The representative electrophoresis of PCR products of the 372 T/C polymorphic region of *TIMP1* gene separated by 8% polyacrylamide gel. Lines: M – DNA marker (200 bp); 1, 3, 7 – homozygote T/T; 2, 6, 8, 9 – heterozygote TC; 4, 5 – homozygote CC.

parameter between 2 groups. An analysis of variance test was used to identify parameters that would make significant differences between more than 2 groups; Scheffe's test was then used to assess the significance of difference in each identified parameter between any 2 groups. STATISTICA 6.0 software (Statsoft, Tulsa, OK, USA) was used to perform analyses.

Results

The genotype and allele frequency and odds ratios of the *MMP1*-1607 1G/2G and the *TIMP1* 372 T/C gene polymorphisms in open angle glaucoma patients and controls are displayed in Tables 3 and 4. The observed genotype frequency of *MMP1* (p>0.05; χ^2=1.68) as well as *TIMP1* (p>0.05; χ^2=3.36) in the controls group were in agreement with Hardy-Weinberg equilibrium (HWE).

The genotype and allele frequencies of the *MMP*1-1607 1G/2G gene polymorphism in POAG patients and the controls group are displayed in Table 3. There was a significant difference in *MMP1* genotype distribution between POAG patients and controls (χ^2=7.15, p=0.028). There were 32% of 1G/1G homozygote, 38% of 1G/2G heterozygote and 30% of 2G/2G homozygote among POAG patients as compared to 36% of 1G/1G homozygote, 44% 1G/2G heterozygote and 20% of 2G/2G homozygotes of controls. The frequencies of the *MMP*1-1607 1G/2G allele were 51% 1G and 49% 2G in POAG as compared to 58% 1G and 42% 2G in controls. We indicated a statistically significant difference in frequency of the 2G/2G genotype of *MMP1* in POAG patients in comparison to the control group (p=0.019; OR=1.73; 95% CI=1.05–2.86). Thus, individuals who were homozygous for the 2G allele presented a significant 1.74-fold increased risk of POAG. We also found a statistically significant increase of the 2G allele presence in POAG patients compared to the control group (p=0.017; OR=1.34; 95% CI=1.03–1.75).

The genotype and allele frequencies of the *TIMP1* 372 T/C gene polymorphism in POAG patients and the controls group are displayed in Table 4. There was no significant difference in *TIMP1* genotype distribution between POAG patients and controls (χ^2=4.108, p>0.05). There were 37% of T/T homozygote, 35% of T/C heterozygote and 28% of C/C homozygote among POAG patients as compared to

Table 3. The genotype and allele frequency and odds ratios (OR) of the *MMP1*-1607 1G/2G polymorphism in open-angle glaucoma (POAG) patients and controls.

Genotype or allele	POAG patients n=196	Frequency	Control subjects n=253	Frequency	OR (95% CI)	p
1G/1G	63	0.32	91	0.36	Ref	Ref
1G/2G	74	0.38	113	0.44	0.95 (0.61–1.46)	0.444
2G/2G	59	0.30	49	0.20	**1.73 (1.05–2.86)**	0.019
1G	200	0.51	295	0.58	Ref	Ref
2G	192	0.49	211	0.42	**1.34 (1.03–1.75)**	0.017

Table 4. The genotype and allele frequency and odds ratios (OR) of the *TIMP1* 372 T/C polymorphism in open-angle glaucoma (POAG) patients and controls.

Genotype or allele	POAG patients n=196	Frequency	Control subjects n=253	Frequency	OR (95% CI)	p
TT	73	0.37	88	0.34	Ref	Ref
TC	68	0.35	110	0.43	0.75 (0.48–1.14)	0.110
CC	55	0.28	55	0.23	1.20 (0.74–1.96)	0.264
T	214	0.55	286	0.55	Ref	Ref
C	178	0.45	220	0.45	1.08 (0.82–1.41)	0.305

Table 5. The genotype and allele frequency and odds ratios (OR) of combined genotypes of the *MMP1*-1607 1G/2G and *TIMP1* 372 T/C polymorphisms in open-angle glaucoma (POAG) patients and controls.

Genotype or allele	POAG patients n=196	Frequency	Control subjects n=253	Frequency	OR (95% CI)	p
1G/1GTT	25	0.13	27	0.11	Ref	Ref
1G/2GTT	23	0.12	40	0.16	0.62 (0.29–1.31)	0.144
2G/2GTT	25	0.13	21	0.08	1.29 (0.58–2.84)	0.338
1G/1GTC	16	0.08	31	0.12	0.56 (0.24–1.25)	0.112
1G/2GTC	28	0.14	53	0.21	0.57 (0.28–1.16)	0.085
2G/2GTC	24	0.12	26	0.10	0.99 (0.46–2.17)	0.575
1G/1GCC	22	0.11	16	0.06	1.49 (0.64–3.45)	0.239
1G/2GCC	23	0.12	29	0.11	0.86 (0.40–1.85)	0.422
2G/2GCC	10	0.05	10	0.04	1.80 (0.38–3.03)	0.546

34% of T/T homozygote, 43% T/C heterozygote and 23% of C/C homozygote of controls. We also did not find an association of *TIMP1* and *MMP1* combined genotype with a risk of POAG after gene-gene interaction analysis (Table 5).

Discussion

The main finding of our study is the evidence that *MMPs* gene polymorphisms might be associated with a risk of occurrence of POAG. However, our data is limited to a Polish population and it must be enlarged to a general worldwide examination. It is estimated that over 68 million people suffer from glaucoma worldwide and there may be more than 750,000 people affected in Poland [20]. Its first symptoms are very often difficult to notice; however, in most patients' eyes the resistance to aqueous humor outflow is significantly increased. An elevated plaque-like material and remodeling within the trabecular meshwork (TM) have been linked to elevated intraocular

pressure in POAG patients [21]. Since elevated intraocular pressure remains the most important risk factor, the role of matrix metalloproteinases in pathogenesis of POAG is strongly suggested [3,4]. However, the pathway leading to remodeling of the TM and retinal ganglion cells death is not fully elucidated.

Matrix metalloproteinases play an essential role in the turnover of the extracellular matrix components, thereby affecting their cellular behavior [12]. In the present study the genotype distribution and allele frequencies of the *MMP1*-1607 1G/2G and the *TIMP1* 372 T/C polymorphisms were determined in 196 patients with POAG and 253 control subjects. There were no differences in the genotype and allele distributions and odds ratios of the *TIMP1* polymorphism between patients and controls; however, a statistically significant increase in the 2G/2G genotype (OR 1.73; 95% CI 1.05–2.86; p=0.019) as well as 2G allele frequency (OR 1.34; 95% CI 1.03–1.75; p=0.017) of *MMP1* in POAG patients in comparison to healthy controls were estimated. According to data from the literature, an additional guanine insertion of 2G allele in the promoter region creates a PEA3 consensus sequence next to the AP-1 binding site, resulting in upregulation of *MMP1* gene expression [9,10].

A significantly altered level of MMPs and their inhibitors in the aqueous humor obtained from POAG patients has been reported [13,15]. Ronkko et al. (2007) have shown an elevated level of MMPs in the chamber angle of patients with primary open angle glaucoma in comparison to normal eyes by immunohistochemical staining [22]. Moreover, these results presented an imbalance between total MMPs: MMP1 + MMP-2 + MMP-3 + MMP-9 and their tissue inhibitors, TIMP1 + TIMP-2 + TIMP-3. Furthermore, Ronko's findings indicated a significant excess of MMP1 over its endogenous inhibitor of TIMP1 in tissue samples from POAG patients. An elevated expression of *MMP1* in human optic nerve head astrocytes of POAG has been also estimated in work done by Agapova et al (2001) [23]. Mossböck et al (1999) did not observe any correlation of *MMP1* genotype with POAG in patients from Austria, but a Greek study showed a trend for the -1607 1G/2G polymorphism association with exfoliation glaucoma [24,25]. Finally, we found an association of the 2G/2G genotype with POAG in Polish patients, which is related to *MMP1* overexpression phenotype. This may in part explain an altered TM remodeling in the pathogenesis of open angle glaucoma. Moreover, understanding the impact of gene polymorphisms associated with POAG will allow the design of new, more effective, therapies [26].

Conclusions

In conclusion, we suggest that the -1607 1G/2G polymorphism of *MMP1* gene might be associated with primary open angle glaucoma in a Polish population. However, further *in vivo* study is needed to evaluate biological importance of *MMP*s polymorphisms as a risk factor of POAG.

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