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The promoter of inducible nitric oxide synthase implicated in glaucoma based on genetic analysis and nuclear factor binding

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Purpose: Nitric oxide has many beneficial functions in the human body at the right amounts, but it can also be hazardous if it is produced in amounts more than needed and has therefore been studied in relation to several neurological and non-neurological disorders. In vitro and in vivo studies demonstrate a connection between the inducible form of Nitric Oxide Synthase, *iNOS*, and the neuropathological disorder glaucoma, one of the major causes of blindness in the world. In this study, we sought to establish the genetic association between *iNOS* and primary open angle glaucoma, POAG, and to find the functional element(s) connected with the pathogenesis of the disease.
Methods: Two microsatellites, 1 insertion/deletion, and 8 single nucleotide polymorphisms (SNPs) in the regulatory region of *iNOS* were genotyped in 200 POAG patients and 200 age-matched controls. Also, the CCTTT-microsatellite was examined for its protein-binding capability in an electrophoretic mobility shift assay, EMSA.
Results: There was a significant difference in allele distribution of the CCTTT-microsatellite, between patients and controls. $(CCTTT)_{14}$, which has been reported to have a higher activity in a reporter-construct, was significantly more abundant in POAG patients, while $(CCTTT)_{10}$ and $(CCTTT)_{13}$ were less common. In EMSA, the $(CCTTT)_{14}$ allele exhibited specific binding of nuclear proteins.
Conclusions: These results, together with other studies on this gene and the CCTTT-microsatellite, establish, for the first time, a genetic association of *iNOS* with POAG and suggest a regulatory function for the microsatellite.

Glaucoma is a general term for a heterogeneous group of ophthalmic disorders involving retinal ganglion cell (RGC) death, optic nerve damage, and visual field loss. It is one of the common causes of visual impairment and blindness in the world and is more common in the elderly [1]. One type of this genetically complex disorder (with the largest number of patients) is primary open angle glaucoma (POAG) with late age at onset and elevated intraocular pressure (IOP) in most cases. Raised IOP can lead to apoptosis and loss of retinal ganglion cells and therefore is an established risk factor for glaucoma [2]. In vitro studies demonstrate that elevated hydrostatic pressure, equivalent to that in glaucoma, induces the expression of *iNOS* in astrocytes [3] in the human eye through the epidermal growth factor receptor [4] and that an inhibition of the *iNOS* gene in vivo leads to protection of RGC in rats with chronic glaucoma [5].

Nitric oxide (NO) is an important and multifunctional yet duplicitous molecule in the human body. It is involved in several processes such as neurotransmission [6], regulation of vascular tone, vasodilatation [7], and regulation of apoptosis [8], and has been implicated in Alzheimer's disease [9] and Parkinson's disease [10]. *NOS1* (*nNOS*), *NOS2A* (*iNOS*), and *NOS3* (*eNOS*) code for the three isoforms of NOS, which produce nitric oxide by oxidation of L-arginine [11]. *NOS1* and *NOS3* are constitutive forms with post-translational regulation and small amounts of NO produced. *iNOS* is the "emergency button", which means that the gene is induced only under conditions where a high amount of NO is required (e.g., during cellular stress, microbial attack, and development of tumors). There are diverse ways of induction, each being specific for a certain cell type or tissue. Cytokines [12] and pressure [13] are two of the recognized inducers of transcription, exerting their effect through an intricate network of transcription factors. Numerous cytokine responsive elements have been found in the promoter and regulatory region of *iNOS*, with a number of them residing in the region upstream of -4 kb from the transcriptional start site [14-16].

Today, for understanding the mechanism behind complex diseases, polymorphisms in noncoding sequences of genes are studied rather than variations in the coding sequences [17]. It is believed that differences in noncoding sequences may influence the transcriptional activity of genes, which in turn result in quantitative trait variations among individuals or in diseases [18]. Such polymorphisms can either be in linkage disequilibrium with a disease-causing element or be the cause of disease themselves. Microsatellites are one kind of these polymorphisms, whose role in susceptibility to disorders and in variations in quantitative traits is not fully understood nor accepted, this in spite of studies that demonstrate the participation of microsatellites in regulation of genes [19-23]. One other example is a pentanucleotide polypyrimidine microsatellite, CCTTT (rs3833912) [24], at position -2.6 kb in the promoter of *iNOS*, which has been a focus of various studies where the authors examine this microsatellite in relation to diverse diseases [25]. Examples of these diseases are malaria [26,27], hypertension [28], diabetic retinopathy [29]

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and nephropathy [30], dementia with Lewy bodies [9], and atopy [31]. Warpeha et al. [29] have investigated whether there is a difference in inducibility of *iNOS* depending on the number of repeats for CCTTT and find that in DLD-1 cells the $(CCTTT)_{14}$ allele has a greater activity than the $(CCTTT)_9$, $(CCTTT)_{12}$, and $(CCTTT)_{15}$ alleles [29]. In contrast, other studies state that a deletion of the microsatellite does not affect the transcription of the gene [15,32].

A more common type of polymorphism, investigated in relation to human disorders, is the single nucleotide polymorphism (SNP). There are two approaches for identification of disease causing elements with the help of SNPs. One is to study a particular SNP for its association with the disorder; the other is to construct haplotypes of SNPs residing in the studied region or gene. SNPs in the regulatory region of *iNOS* have been extensively studied in relation to malaria, where a possible association is found [26,33].

Inducible nitric oxide synthase has been examined in several in vitro and in vivo studies for its role in glaucoma [34,35]; hitherto, no genetic association study has been reported. The aim of the present case-control study was to establish a possible genetic association between glaucoma and *iNOS* and ascertain the role of polymorphisms in onset and/or progression of glaucoma. Regulatory sequences of this gene were therefore resequenced and polymorphisms were genotyped in a patient-control study. The results provide evidence for an association of *iNOS* to glaucoma and suggest a possible role for the CCTTT-microsatellite in the progression of this disorder.

TABLE 1. SEQUENCE OF PRIMERS USED FOR STUDY OF POLYMORPHISMS

Primer name	Direction	Position	Sequence (5'-3')
PCR primers			
C/T	Forward	-3761	TCCAGGATAATGAGCCCAAG
	Reverse		GTCCCACCACAAACACCTG
C/T	Forward	-3031	AAAAACCATTGCATTTTACACTTT
	Reverse		TGGCAAGCTGTGAGAAGGTA
C/G	Forward	-2441	GCTCTCTGCAGCCTCTAACTC
	Reverse		AGCCAGCTGGGAAGGAAATA
C/T	Forward	-1659	TCTTGGGTGGGGGCATTA
	Reverse		CATCTTCCCTTCAGGCTTCA
G/T	Forward	-1026	TCCCTTTGCTTCTCAACTTCTC
	Reverse		TGGAGTCTCATTCTGTCACCA
A/G	Forward	-277	AGCTTCCTGGACTCCTGTCA
	Reverse		GGAGCCTCAGTTTTCGACTC
C/T	Forward	1211	GATCTCAGATTTCGAGGGATGA
	Reverse		ATGGCTTTACAAAGCAGGTCA
C/T	Forward	1638	GATCTCAGATTTCGAGGGATGA
	Reverse		ATGGCTTTACAAAGCAGGTCA
Sequencing primers			
CCTTT-	Forward	-2600	ACCCCTGGAAGCCTACAACTGCAT
microsatellite	Reverse		GCCACTGCACCCTAGCCTGTCTCA
TAAA-repeat	Forward	-751	ACTCCGCTCCAGTCTTGGT
	Reverse		AGGAAGGCTATTGGCACAGA
GTGTGTT	Forward	-453	TCTGTGCCAATAGCCTTCCT
ins/del	Reverse		TCCCTGTCCATCCTCTCACT
SNaPshot primers			
C/T	Forward	-3761	ATCAGAAGAGGCCAGGAATG
C/T	Forward	-3031	(GATC)(2)CAGTGAAGCTGTGCCTCGAA
C/G	Reverse	-2441	(GATC)(4)GAAAATAAATTAGCTGGGTATG
C/T	Forward	-1659	(GATC)(6)TTCCTTGAACAAGGCAGAAC
G/T	Reverse	-1026	(GATC)(3)GGATTACAAGGGTTAGCCAC
A/G	Forward	-277	(GATC)(2)GGCTCCGTGGTGCCTCT
C/T	Forward	1211	(GATC)(7)TTGAGTGCTTTCCCCAGTAA
C/T	Forward	1638	(GATC)(10)CCTTCCCTCACCTCTGCTTC

The table below summarizes all primers used in the study of polymorphisms for PCR, sequencing, and SNaPshot™. The column “Primer name” refers to the type of polymorphism and “Position” is its location in relation to transcriptional start position at +1. The sequence for the CCTTT-microsatellite primer was taken from [25]. For SNaPshot™ only one primer is required for each SNP. In the “Sequence” column for the SNaPshot™ primers, the numbers in parentheses indicate the number of “GATC” repeats.

METHODS

Subjects: The individuals in this study were recruited from the glaucoma clinic at the Department of Ophthalmology, University Hospital, Uppsala, and the Department of Ophthalmology, Tierps Hospital, Tierp, Sweden. Criteria for the selection were increased IOP in at least one eye and glaucomatous damages to optic nerve head and/or glaucomatous damage to the visual field. Peripheral blood from 200 patients was collected. Out of these, 99 were male with an average age at first diagnosis of 65.1 (range 27-90) and 101 female with an average of 66.5 (range 39-91). In total, 50 had at least one case of glaucoma in the family. The mean pressure for the right eye of patients was 30.9 (range 18-57, median 29) and for the left eye 31.3 (range 19-59, median 30). The threshold for elevated IOP was 24. Moreover, 200 samples were collected from control individuals matched with the patients for age, sex, geographic, and ethnic origin. Glaucoma was excluded in this group by measuring IOP and examining the optic disc (by ophthalmoscopy). None of the studied individuals was related. The Random Population samples were DNA from blood donors in Uppsala. There was no information regarding age or geographic and ethnic origin of the donors, nor about the health condition of these individuals.

Informed consent was obtained from all participants. This study was approved by the local Research Ethics Committee at Uppsala University and performed according to the Declaration of Helsinki.

DNA preparation and genotyping: DNA was prepared by standard procedures from peripheral blood leukocytes.

All length polymorphisms and their flanking sequences were amplified with fluorescently (FAM) labeled primers (GensetOligos/Proligo, France) on a PTC-225 MJ Research, applying regular PCR-protocols. Primer sequences are presented in Table 1. The amplicons were analyzed on an ABI Prism™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Size of the fragments was established by sequence analysis of reference samples followed by genotyping these and all other samples using the standard computer programs GeneScan® version 3.1 and Genotyper® version 2.5 (Applied Biosystems). Reanalyzing 46 randomly chosen samples from the patient and control groups confirmed the result.

Genotyping of the SNPs was done with SNaPshot™, a method based on the mini sequencing, Multiplex kit from ABI PRISM®. For each 10 µl reaction, 3 µl of the purified PCR-product was mixed with 1 µl of a primer mix (2.0 µM of each primer) and 1-2 µl of the SNaPshot™ mix. Otherwise, the protocol provided with the kit was followed. Sequences of the PCR- and SNaPshot™-primers are presented in Table 1.

Sequencing: All sequencing was done on MegaBACE™ 1000 (Molecular Dynamics and Amersham Lifescience, Uppsala, Sweden) with sequences prepared with DYEnamic™ ET dye terminator cycle sequencing kit from Amersham Biosciences (Piscataway, NJ). In short, the fragments were amplified by PCR and purified by SAP-Exo I treatment. After the sequencing reaction, the products were precipitated and purified and the sequences were read with MegaBACE™. Results were analyzed on Sequencher™ version 4.1.1 (Gene Codes, Ann Arbor, MI).

Electrophoretic mobility shift assay (EMSA): HeLa nuclear extracts were purchased from Promega (Madison, WI). The general EMSA protocol provided with the Gel Shift Assay Core System kit (Promega) was followed with slight modifications.

Oligonucleotides (GensetOligos/Proligo, France) constituting both strands of $(CCTTT)_{14}$-microsatellite corresponding to the $(CCTTT)_{14}$ allele were synthesized and annealed to get double stranded (DS) products at a final concentration of 1.75 pmol/µl. Only the microsatellite and no flanking sequences were used. DS oligonucleotides were labeled with (γ^{32}P) ATP (3,000 Ci/mmolat 10 mCi/ml).

Single stranded $(CCTTT)_{14}$ and $(AAAGG)_{14}$ was annealed to obtain double strands and treated with Klenow-fragment (Invitrogen, Carlsbad, CA). The product was loaded on a 4.5% NuSieve® GTG® agarose (Cambrex, Rockland, ME) in 1X

Table 2. Allele distribution of CCTTT-microsatellite in POAG patients, age-matched controls, and random population

Number of repeats	POAG patients: Allele frequency	POAG patients: Number of homozygotes	Age-matched controls: Allele frequency	Age-matched controls: Number of homozygotes	Random population: Allele frequency	Random population: Number of Homozygotes
8	4 (0.0100)	0 (0.00)	2 (0.0050)	0 (0.00)	2 (0.0049)	0 (0.00)
9	17 (0.0425)	0 (0.00)	9 (0.0225)	0 (0.00)	15 (0.0369)	0 (0.00)
10	37 (0.0925)	1 (0.0050)	55 (0.1375)	5 (0.0250)	58 (0.1429)	6 (0.0148)
11	81 (0.2025)	9 (0.0450)	83 (0.2075)	10 (0.0500)	73 (0.1798)	7 (0.0172)
12	160 (0.4000)	29 (0.1450)	151 (0.3775)	30 (0.1500)	137 (0.3374)	24 (0.0591)
13	42 (0.1050)	3 (0.0150)	65 (0.1625)	8 (0.0400)	78 (0.1921)	10 (0.0246)
14	41 (0.1025)	3 (0.0150)	22 (0.0550)	1 (0.0050)	33 (0.0813)	6 (0.0148)
15	9 (0.0225)	0 (0.00)	6 (0.0150)	0 (0.00)	7 (0.0172)	0 (0.00)
16	8 (0.0200)	0 (0.00)	6 (0.0150)	0 (0.00)	2 (0.0049)	0 (0.00)
17	1 (0.0025)	0 (0.00)	1 (0.0025)	0 (0.00)	1 (0.0025)	0 (0.00)
Total	400	45	400	54	406	53

The allele frequency and number of homozygotes for each allele is presented in numbers and percentage (in parentheses) compared between primary open-angle glaucoma (POAG) patients, age-matched controls, and the random population. The column "Number of repeats" refers to how many times the CCTTT is repeated without any interruption (the perfect repeat).

TAE together with a size marker. The oligonucleotide was visualized on a UV table and a defined band was cut out of the gel. DNA was purified on a GFX™ column (Amersham Biosciences, Buckinghamshire, England) and labeled as above. To investigate binding properties of the microsatellite, different reactions were performed using DS $(CCTTT)_{14}$ as a model. The negative reaction contained only labeled DS oligonucleotide and poly d(I-C), whereas the positive reaction contained labeled DS oligonucleotide, poly d(I-C), and HeLa nuclear extract. The specific competitor reaction was performed as the positive reaction including 50x excess of unlabeled DS oligonucleotide, while the unspecific competitor reaction included 50x excess unlabeled Sp1 oligonucleotide in addition to the material in the positive reaction. For supershift, 0.2 μg of antibody was added and the sample was incubated 30-45 min in room temperature. Applied antibodies were for PTB (hnRNPI N-20), hnRNPK (H-300), and AP-2α (3B5) purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Final volume of each mix was 10 μl. Samples were loaded on a native 4% polyacrylamide gel and electrophoresed at 350 V for 30-45 min. Products were analyzed using Fuji Film BAS-1800 II Phosphoimager instrumentation with exposure times varying from 2 h to overnight. Where mentioned, 1,10-Phenantroline (Sigma, England) was added to a positive reaction (see above) to a final concentration of 3.5 or 5 nmols in 10 μl.

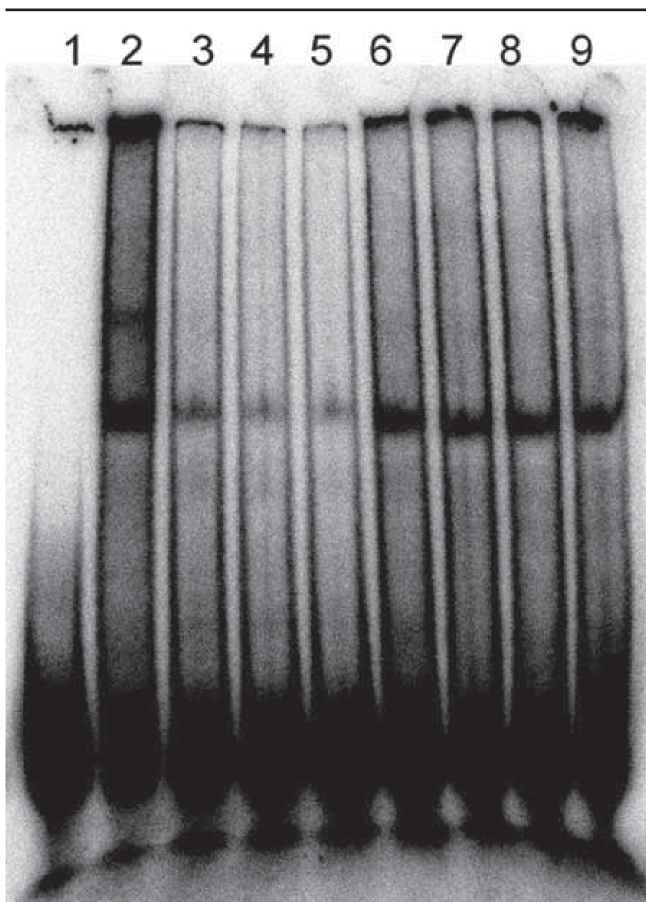

Figure 1. Specific protein binding by the DS $(CCTTT)_{14}$ allele of the microsatellite in the *iNOS* promoter. An EMSA was performed with Klenow-fragment treated, labeled, double stranded $(CCTTT)_{14}$ oligonucleotide, incubated with HeLa nuclear extract to examine nuclear factor binding capability of the microsatellite. Lane 1: Negative reaction containing labeled DS $(CCTTT)_{14}$ oligonucleotide. Lane 2: Positive reaction with labeled DS $(CCTTT)_{14}$ oligonucleotide and 1.5 μl of HeLa nuclear extract (concentration of stock >2.4 μg/μl). Lanes 3-5: Specific competitor reaction with same components as Lane 2 with 10x, 25x, and 50x excess of unlabeled DS $(CCTTT)_{14}$ oligonucleotide added. Lane 6: A competitor reaction with 50x excess of unlabeled Sp1 oligonucleotide. Lanes 7-9: Supershift with 0.2 μg of antibody against PTB, hnRNPK, and Ap2.

Haplotype reconstruction: All SNP-genotypes for the patients and controls were made as one input file for the haplotype reconstruction program PHASE version 1.0 (Oxford University, England) [36,37]. The frequency of each haplotype for each group was counted using the output data. In addition, the total haplotype, including SNPs, microsatellites, and the insertion/deletion for a material with parent-offspring trios were reconstructed separately. These were corrected manually by comparing the offspring haplotypes with their parents.

Statistical analysis: The χ^2 test applied on the genotyping results was the two way contingency table in STATView® version 4.5 (Abacus Concepts, Cary, NC). An α level of 0.05 was chosen.

RESULTS

Selection of study subjects: For a genetic study of primary open angle glaucoma (POAG), 200 patients were collected based on two inclusion criteria; (1) glaucomatous damage to the optic nerve head (ONH) and/or glaucomatous damage to the visual field, and (2) increased IOP. Thus, all the individuals included in the study group were high tension glaucoma patients. These were matched by age, sex, geographic, and ethnic origin to 200 control individuals in whom glaucoma was excluded by IOP measurements and examiniation of the optic disc.

Genotyping of the CCTTT-microsatellite reveals a difference: The initial step of this work was to genotype the CCTTT-microsatellite in the POAG patients and age-matched controls to determine the allele frequencies of this polymorphism in the Swedish population. The distribution of the alleles was essentially in concordance with previous studies in the European population, with the peak at the $(CCTTT)_{12}$ (Table 2) [38]. Comparing POAG patients with age-matched controls indicated a difference in overall distribution of alleles (χ^2=18.456, df=7, p=0.0101). To corroborate this, a group of 204 randomly chosen individuals, called "Random population", was genotyped for the microsatellite. Correspondingly, a difference could be detected between the POAG patients and the "Random population" (χ^2=21.167, df=6, p=0.0017). There was no difference in allele frequency between age-matched controls and "Random population" (χ^2=6.416, df=6, p=0.3783). Furthermore, among POAG patients the $(CCTTT)_{14}$ had a higher frequency (χ^2=6.220, Fisher's exact p=0.0175), and the $(CCTTT)_{13}$ had a lower frequency (χ^2=5.707, Fisher's exact p=0.0220) compared to age-matched controls, while

compared to "Random population", the $(CCTTT)_{10}$ and $(CCTTT)_{13}$ were less frequent (χ^2=4.194, Fisher's exact p=0.0290; χ^2=12.068, Fisher's exact p=0.0005; respectively).

Nuclear factors bind to the microsatellite: $(CCTTT)_{14}$ has been demonstrated to enhance the activity of a minimal *iNOS* promoter more than three other alleles of the same microsatellite, upon induction by IL-1β [29]. One way of mediating such an effect is through binding of nuclear factors. This was studied in an electrophoretic mobility shift assay (EMSA) with a labeled double stranded $(CCTTT)_{14}$ oligonucleotide. Repeat-oligonucleotides anneal in a way so that double strands with single strand overhang, and possibly other alternative structures, along with the regular double strands may form. Therefore, to ensure that the nuclear factor was binding to DS-oligonucleotides, single stranded $(CCTTT)_{14}$ and $(AAAGG)_{14}$ oligonucleotides were annealed and then treated with Klenow-fragment. Klenow-fragment can elongate partially single-stranded oligonucleotides to double strands and has an exonuclease activity that removes single strands from 3' to 5'. This would result in a pool of double stranded oligonucleotides with somewhat differing sizes. The reaction was then analyzed on a 4.5% agarose gel, a defined band was excised, and DNA was isolated by extraction. When analyzed by EMSA, binding of nuclear factors to the DS oligonucleotide was detected, which was competed by DS unlabeled (CCTTT), but not a Sp1-oligonucleotide (Figure 1). Our unpublished data indicate a similar specific binding of nuclear factors by other alleles of this microsatellite. Polypyrimidine tract binding protein (PTB) has been suggested to interact with single strand pyrimidine containing sequences [39]. However, using an antibody against PTB did not lead to a supershift and PTB can therefore be excluded as a candidate for binding to the double stranded (CCTTT) microsatellite.

Investigating the interacting factor(s): An attempt was made to identify protein(s) binding to the microsatellite. The web-based program, MatInspector version 2.2 [40], was used to predict possible candidates, where NFAT and GKLF were indicated as most probable binders. A third candidate was GABP, which belongs to ETS family of transcription factors, with CCTTN as their core binding sequence. All three factors were evaluated in EMSA using specific DS oligonucleotides with the consensus sequence for each factor. NFAT did not seem to be expressed in HeLa cells, while neither GKLF- nor GABP-binding was abrogated by cold $(CCTTT)_n$-oligonucleotide (data not shown).

To restrict the number of probable candidates, an EMSA was performed using 1,10-phenantroline, which is a potent Zn-chelator, preventing DNA from interacting with zinc finger proteins. 1,10-Phenantroline treatment completely abol-

Table 4. Occurrence of SNP-haplotypes in POAG patients and age-matched controls

Identifier	Haplotype	POAG patients (%)	Age-matched controls (%)
1	CCCCGATC	156 (0.390)	153 (0.383)
2	CCCCGACT	84 (0.210)	87 (0.218)
3	TCGCTGCC	69 (0.173)	64 (0.160)
4	CTGTTGCT	38 (0.095)	30 (0.075)
5	CCGCGGCC	18 (0.045)	38 (0.095)
6	CCCCGACC	13 (0.033)	13 (0.033)
7	CTGTTGCC	13 (0.033)	5 (0.013)
8	TCGCTGTC	2 (0.005)	5 (0.013)
9	Other	7 (0.018)	5 (0.013)
Total		400	400

Frequency and percentage of each haplotype is presented for the primary open-angle glaucoma (POAG) patients and controls. The order of SNPs in each haplotype is from 5' (left) to 3' (right) of the regulatory sequences and 5'-UTR. Haplotypes with a frequency less than 1% in both groups are summarized as "other".

Table 3. SNP-allele frequencies in POAG patients and age-matched controls

			POAG patients		Age-matched controls	
rs-number	Position	SNP	Number of alleles (%)	Homozygotes	Number of alleles (%)	Homozygotes
2779251	-3761	C	328 (0.820)	134	330 (0.825)	137
		T	72 (0.180)	6	70 (0.175)	7
11080358	-3031	C	349 (0.873)	154	365 (0.913)	166
		T	51 (0.128)	5	35 (0.088)	1
2779250	-2441	C	258 (0.645)	84	255 (0.638)	80
		G	142 (0.355)	26	145 (0.363)	25
8078340	-1659	C	349 (0.873)	154	365 (0.913)	166
		T	51 (0.128)	5	35 (0.088)	1
2779249	-1026	G	275 (0.288)	94	293 (0.733)	105
		T	125 (0.313)	19	107 (0.268)	12
2779248	-277	A	257 (0.643)	83	254 (0.635)	79
		G	143 (0.358)	26	146 (0.365)	25
6505483	1211	C	240 (0.600)	75	240 (0.600)	73
		T	160 (0.400)	35	160 (0.400)	33
3730013	1638	C	274 (0.685)	98	280 (0.700)	98
		T	126 (0.315)	24	120 (0.300)	18

Frequency of each SNP-allele is compared between primary open-angle glaucoma (POAG) patients and controls. Allele frequency and number of homozygotes for each allele is presented in numerals and per cent (in parentheses). rs-numbers were obtained from the International HapMap Project web site. Position is relative to transcriptional start site.

ished binding of Sp1 to its specific DS oligonucleotide, which is in agreement with the fact that Sp1 is a Zn-finger protein. This was not the case when the reagent was used in a similar assay with (CCTTT) DS oligonucleotides and HeLa extract (data not shown). The results therefore suggest that the protein(s) binding the microsatellite does not contain a zinc finger domain.

Polynucleotide polymorphisms are equally distributed: The bi-allelic microsatellite $(TAAA)_{3/4}$ (GeneCard number rs12720460), at position -752 [41], in the *iNOS* promoter is reported to have protein binding capacities and differential regulatory activity depending on the allele [42]. In this study, there was no difference in allele frequencies for this polymorphism or for the insertion/deletion (GTGTGTT; GeneCard number rs1799765) at position -453, among POAG patients and age-matched controls (data not shown).

Single nucleotide polymorphisms in the iNOS *regulatory region:* To identify other genetic variants in *iNOS*, 4 kb upstream of the transcriptional start site, exon 1, and intron 1 of the promoter were resequenced in 12 individuals. This led to the finding of 8 SNPs over a 5,700 bp sequence, out of which 4 had not been reported in other studies at the time of this experiment. These were then genotyped by SNaPshot™. No difference in SNP-allele frequencies could be detected between POAG patients and age-matched controls (Table 3).

Haplotypes of the SNPs were constructed using PHASE (Table 4) [36,37]. Although no difference in overall haplotype distribution could be noted, SNP-haplotype 5 was found to be less frequent in POAG patients (χ^2=7.680, Fisher's exact p=0.0056).

Due to the high number of alleles, PHASE could not include the CCTTT-microsatellite in the haplotypes. Genotyping of all polymorphisms investigated in this study, in nuclear families, verified that each CCTTT allele is present on several SNP-haplotypes, (as expected the repeat polymorphism shows evidence of higher mutation rate than the SNP-haplotypes; data not shown).

DISCUSSION

In this study, we provide evidence of a genetic association between *iNOS* and glaucoma based on genotyping of the CCTTT-microsatellite in the inducible nitric oxide synthase regulatory sequence, where a difference in the overall distribution of alleles was discovered between primary open angle glaucoma patients and age-matched controls. Furthermore, our EMSA study revealed the capability of nuclear protein binding for this microsatellite. Even though the specificity of this binding was proven, additional EMSAs are required for characterizing the nuclear factor. As mentioned before, a previous study demonstrates a higher transcriptional activity for $(CCTTT)_{14}$ compared to alleles $(CCTTT)_9$, $(CCTTT)_{12}$, and $(CCTTT)_{15}$ [29]. This is in agreement with our data, indicating $(CCTTT)_{14}$ as one of the possible risk factors for primary open angle glaucoma, although the genetic effect is most likely more complicated with other alleles of this repeat involved. Since there was no overall difference in SNP-haplotype distribution between POAG patients and age-matched controls, we favor the hypothesis that the association is mediated by the CCTTT-microsatellite rather than the SNPs.

Primary open angle glaucoma is a complex disease associated with several loci and genes [43]. Thus far, no common variant has been reported to be associated with this disorder, making it an example of a common disease with many rare variants contributing to its pathogenesis. Functional studies of *iNOS* have indicated that this gene is induced in high pressure glaucoma [3] and is contributing to the disease [44], making it possible that inherited predisposition to glaucoma could be mediated by this gene. However, a recent study [45] has not been able to confirm such a functional association. While our study shows an association between this gene and POAG, there is obviously a need of extensive experiments to establish the role of *iNOS* in pathogenesis of this disorder.

We hypothesize that the CCTTT-microsatellite may be involved in the regulation of *iNOS* in either (or a combination) of two ways. For one thing, it may function as an enhancer. Currently two characteristics define enhancers; (1) the ability to bind nuclear factors and (2) the independency of orientation or distance relative to the transcriptional start site [46]. The CCTTT-microsatellite clearly exhibited nuclear factor binding in our study. Furthermore, in the Warpeha et al. [29] study, the microsatellite was transferred from its original position, at 2.6 kb upstream of transcriptional start site, to the 5'-end of a 1.2 kb fragment of the *iNOS* promoter, where it was able to enhance transcription of the construct with different strength depending on the allele tested. A second possible function of the microsatellite is as a spacer element. In *E. coli* the length of the spacer region between -10 and -35 boxes in the promoter have been suggested to be more important for regulation than the actual sequence [47], a model that can be partially applicable for microsatellites in promoters of human genes. Increased spacing by a tandem repeat may create flexibility in the DNA-chain, facilitating for interactions between sequences that flank the repeat. The $(CCTTT)_{14}$ might be an adequate spacing (70 bp) for bringing nuclear factor binding elements, immediately upstream of the microsatellite, closer to the proximal promoter leading to the high activity reported for this allele. One should also consider that the microsatellite is in a position that is approximately halfway between several important NF-κB binding sites located at 5-6 kb upstream of the transcriptional start site [15] and the transcriptional initiation site. Deletion of a large sequence, including the microsatellite, between the response elements and the core promoter as in the two reported studies [15,32], may fulfill the requirements for bringing the distal sequences closer and thereby eliminating the need for a spacer element.

In this study, frequency of single nucleotide polymorphisms and their haplotypes in the Swedish population was also determined. Although no difference in frequency of SNPs or total distribution of haplotypes could be found, haplotype 5 was found to be less abundant among patients. Whether a part of this haplotype is directly involved or in linkage disequilibrium with a functional element in the promoter is unknown. It is possible that different alleles of a certain SNP in a promoter affect the transcription factor-binding capacity of the response

element and thereby play a role in the regulation of a gene. For example, *CHGA* haplotypes are demonstrated, by reporter activity assay, to alter level of expression of the gene [48]. In addition, haplotypes of *iNOS* are related to other diseases including malaria [33] and hepatitis C virus infection [49]. Even though the functionality of these haplotypes for *iNOS* has not been evidenced by reporter assay, the C nucleotide at position -1659 displays binding of more protein than the T-allele, in an EMSA study [33]. For a better understanding of how the different SNPs and the haplotypes of *iNOS* promoter affect the expression of the gene, more extensive functional studies are required [50].

In conclusion, we provide evidence of an association between *iNOS* and glaucoma and for the possible role of CCTTT-microsatellite in the pathogenesis of this disease. A look at the published sequences of the human genome reveals a gene desert of about 75 kb upstream and 100 kb downstream of the exons of *iNOS*. Gene deserts may contain regulatory elements affecting the expression of nearby genes [51]. A study of the polymorphisms and other regulatory elements in the flanking sequences of the exons will give a more complete and accurate picture of the elaborate regulation of inducible nitric oxide synthase.

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