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Investigations on the association between normal tension glaucoma and single nucleotide polymorphisms of the endothelin-1 and endothelin receptor genes

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Purpose: In normal tension glaucoma (NTG), intraocular pressure is within normal the range; thus, some mechanism other than increased pressure contributes to the optic neuropathy. Endothelin may be an important contributor to the development of the optic neuropathy characteristic of glaucoma. We investigated whether polymorphisms of the endothelin-1, endothelin receptor type A, and endothelin receptor type B genes were associated with NTG.
Methods: Sixty-seven Korean NTG patients and 100 healthy Korean subjects were enrolled. DNA from peripheral blood leukocytes was extracted and genotype distributions of six polymorphisms in genes encoding endothelin-1 (*EDN1:c.-131dupA*, *EDN1:c.594G>T*), endothelin receptor type A (*EDNRA:c.-231G>A*, *EDNRA:c.*70C>G*, *EDNRA:c.*1222C>T*), and endothelin receptor type B (*EDNRB:c.831A>G*) were determined. Genotype and allele distributions were compared between patients and controls. In NTG subjects, untreated baseline intraocular pressure and age at the time of diagnosis, as well as the mean deviation and pattern standard deviation values of automated static perimetry were examined for an association with these genetic polymorphisms.
Results: The polymorphism of *EDNRA:c.*1222C>T* was significantly associated with NTG (p=0.028, OR=3.33, 95% CI 1.05-10.24). In addition, the AA genotype of the *EDNRA:c.-231G>A* polymorphism was associated with a lower baseline intraocular pressure than in the GG+GA genotype group (14.0±2.8 mm Hg versus 16.2±2.3 mm Hg, p=0.047). No polymorphism was associated with visual field parameters.
Conclusions: A polymorphism of the endothelin receptor type A gene is associated with NTG.

Glaucoma, which is thought to be a chronic neurodegenerative disease, is the second most common cause of blindness worldwide [1,2]. Normal tension glaucoma (NTG), a subset of glaucoma, is an entity with progressive glaucomatous optic neuropathy and corresponding visual field defects, but with intraocular pressure (IOP) in the statistically normal range. Thirty-seven percent of patients with NTG have a family history of glaucoma, and its higher prevalence in Korean (2.04%) and Japanese (3.6%) populations than in the Caucasian population (0.6%) suggests that there might be a genetic predisposition for NTG [3-6]. Population based association studies using polymorphisms of candidate genes offer one approach to identify which genes might confer increased susceptibility to this form of glaucoma.

Endothelin-1 (EDN1), one of the most potent physiologic vasoconstrictors currently known, has been shown to play an important role in the pathophsiology of glaucoma through its various actions, including reduction of blood flow to the optic nerve head [7]. Endothelin receptor type A (EDNRA) and endothelin receptor type B (EDNRB) are distinct receptors that mediate the biological effects of EDN1. Recently, EDNRA and EDNRB have been located in the region of the optic nerve head [8]. The stimulation of EDNRA evokes marked and sustained vasoconstriction of vascular smooth muscle cells [9]. In contrast, in endothelial cells, stimulation of EDNRB leads to release of nitric oxide in some blood vessels, evoking transient vasodilation [10]. A recent study has suggested that the GG genotype of *EDNRA:c.*70C>G* polymorphism is associated with more severe visual field defects in Japanese NTG patients, although genotype distributions of *EDN1*, *EDNRA*, and *EDNRB* were not associated with open-angle glaucoma [11].

In this study, we investigated whether polymorphisms of the *EDN1*, *EDNRA*, and *EDNRB* genes were associated with NTG in the Korean population. Compared with the Caucasian population, the Korean population should be a better ethnic group for genetic studies related to NTG because of its single ethnic origin and the higher prevalence of NTG.

METHODS

Patient selection: The study population was composed of 67 patients with NTG and 100 healthy volunteers. All subjects were of Korean origin and were, to our knowledge, unrelated. NTG patients were recruited at the Glaucoma Clinic and volunteers at the Health Promotion Clinic of Seoul National Uni-

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versity Hospital. Written informed consent was obtained from all subjects and the study had the approval of the Institutional Review Board of the Seoul National University Hospital Clinical Research Institute. The diagnostic criteria for NTG were: glaucomatous optic neuropathy with corresponding visual field defects; diurnal IOP persistently below 21 mm Hg without medication; open anterior chamber angle; and absence of any known secondary cause for glaucomatous optic neuropathy. IOP was measured using a Goldmann applanation tonometer and the baseline IOP was defined as the average of diurnal measurements taken every 1.5 h from 9 AM to 6 PM. Visual fields were evaluated using the 30-2 program of the Humphrey visual field analyzer Model 750 (Zeiss Inc., San Leandro, CA) or Model 630 (Allergan Inc., San Leandro, CA). Mean deviation (MD) and pattern standard deviation (PSD) of these visual fields were obtained for the analysis. The first two perimetric results were excluded in order to avoid learning effects, and the next two perimetric results were adopted as baseline values. MD and PSD values were obtained by averaging baseline perimetric values.

Control subjects had a best corrected visual acuity of better than 20/25, an IOP below 21 mm Hg, no suspicious findings of glaucoma in the disc or fundus, no family history of glaucoma, and no evidence of systemic disease such as diabetes and hypertension.

Gene polymorphisms: Six polymorphisms were identified through a literature review [12,13]. They were the *EDN1:c.594G>T* in exon 5 and *EDN1:c.-131dupA* in the 5'-untranslated region (UTR) of the *EDN1* gene; the *EDNRA:c.-231G>A* in the 5'-UTR, *EDNRA:c.*70C>G*, and *EDNRA:c.*1222C>T* in the 3'-UTR of the *EDNRA* gene; and the *EDNRB:c.831A>G*in exon 4 of the *EDNRB* gene. The genotype of the *EDN1:c.594G>T* polymorphism was identified using the restriction fragment length polymorphism method with mutagenic primers. The genotype of *EDNRA:c.*70C>G* was identified using the denaturing high-performance liquid chromatography (DHPLC) method. The others were analyzed using the single nucleotide extension assay. Nomenclature used in this article follows the recommendations from the Human Genome Variation Society [14].

Genomic DNA was extracted from EDTA anticoagulated peripheral blood leukocytes using a Gentra PureGene DNA isolation kit (Gentra System, Inc., Minneapolis, MN). Polymerase chain reactions (PCR) were carried out in a reaction volume of 20 µl using 50 ng of genomic DNA, 1X PCR buffer (10 mM Tris-HCl, pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 uM of each dNTP, 20 pmole of each primer (Table 1), and 0.5 units of *Taq* polymerase (AmpliTaq Gold™; Applied Biosystems, Foster, CA). Cycling parameters were 10 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at primer specific temperature for 30 s (Table 1) and extension at 72 °C for 60 s. Final extension was at 72 °C for 7 min.

In the case of *EDN1:c.594 G>T*, the 184 bp PCR products were digested with *Hin*dIII and electrophoresed in a 2% agarose gel with ethidium bromide. The presence of the G allele caused the 184 bp PCR product to become two fragments of 160 bp and 24 bp. With *EDNRA:c.*70C>G*, the genotype was identified using the WAVE DHPLC system (Transgenomic, Inc., Omaha, NE) at 60.2 °C. The resulting

Table 1.

SNP	Direction	Primer sequence	Annealing temperature (°C)
EDN1:c.-131dupA (exon 1)	Forward	GGCAGAGAGCTGTCCAAGTC	55
	Reverse	CCCAAAGGAAAACGAAGAAA	
	Snapshot	TCAGCCCAAGTGCCCTTT	
EDN1:c.594G>T (exon 5)	Forward	CAGGTTTTGTTTGTGCCAGA	53
	Reverse	GGTCACATAACGCTCTCTGGAAAG	
EDNRA:c.-231G>A (exon 1)	Forward	GGAGAGGCTTCATCCATCC	53
	Reverse	CCCAGTCCCCGATAAGAAAG	
	Snapshot	gactgactgTTCCCCGGCTTCAGAAAAC	
EDNRA:c.*70C>G (exon 8)	Forward	AGTCTGTTCCTTCCCCCAGT	55
	Reverse	TCGTAGATGTTGTGGGTGGA	
EDNRA:c.*1222C>T (exon 8)	Forward	TTTTGAAGTGGCCAGATGAG	55
	Reverse	AAATGCCCAGCAAAAGTCAC	
	Snapshot	gactgactgacCAGAACTTACGATTCTTCACTT	
EDNRB:c.831A>G (exon 4)	Forward	GAAGATAATCATTCCCTGATGAA	55
	Reverse	CAATCTGCATGCCACTTTTC	
	Snapshot	gactgactgactgactGCAAGCAGAAATAGAAACTGAA	

Primers for polymerase chain reaction and single nucleotide extension assay in endothelin-1 (EDN1), endothelin receptor type A (EDNRA), and endothelin receptor type B (EDNRB) polymorphisms.

DHPLC trace profiles were examined using Navigator™ software. The chromatographs were compared with profiles of wild-type DNA fragments. Samples with aberrant profiles were sequenced. In other single nucleotide polymorphisms (SNPs), the PCR products were pooled and subsequently cleaned up through incubation with Exo I and SAP (USB Corp., Cleveland, OH) at 37 °C for 60 min. Each extension reaction, using 3 μl of PCR product, between 0.05 and 0.5 pmoles of each primer (Table 1) and 5 μl of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems), was carried out by repeating the following cycle 30 times: 96 °C for 10 s, 40 °C for 5 s, and 60 °C for 30 s. When completed, 1 U of SAP was added and the reaction mixture was incubated for 60 min. Then, 0.5 μl of reaction product were mixed with 9.4 μl of formamide and 0.1 μl of 120 LIZ dye (Applied Biosystems), and analyzed by ABI Prism 3100 Genetic Analyzer and ABI GeneScan 3.1 analysis software (Applied Biosystems). The design of primers and probes, as well as the positioning of the SNPs relative to the translation initiation site of the genes were based

Table 2.

Characteristics	Patients	Controls	p value
Age (years)	47.1±10.4	49.3±9.2	NS
Gender ratio (men:women)	28:39	47:53	NS
IOP (mm Hg)	16.0±2.4	15.3±2.5	NS
Maximun diurnal IOP	17.3±2.4		
Minimum diurnal IOP	14.5±2.8		
Family history of glaucoma	14 (20.9%)	0 (0%)	
Visual field			
MD (dB)	-6.54±5.45		
PSD (dB)	8.34±4.98		

Clinical characteristics of normal tension glaucoma patients and controls. In the table, IOP indicates intraocular pressure, MD is mean deviation of program 30-2 of Humphrey visual field analyzer, and PSD indicates the pattern standard deviation of program 30-2 of Humphrey visual field analyzer. Statistical testing was done by independent t-test or the χ^2 test.

Table 3.

Gene/polymorphism	Genotype			p value	Odds ratio	Allele		p value
Endothelin-1								
EDN1: c.-131dupA	AA	A-	--	AA+A-/--		A	-	
Patients	46	21	0	0.3		113	21	0.96
Controls	70	29	1			169	31	
EDN1:c.594G>T	GG	GT	TT	GG+GT/TT		G	T	
Patients	31	29	7	0.76		91	43	0.27
Controls	56	35	9			147	53	
Endothelin receptor type A								
EDNRA: c.-231G>A	GG	GA	AA	GG+GA/AA		G	A	
Patients	29	32	6	0.14		90	44	0.49
Controls	44	39	17			127	73	
EDNRA: c.*70C>G	CC	CG	GG	CC+CG/GG		C	G	
Patients	15	38	14	0.32		68	66	0.50
Controls	24	46	30			94	106	
EDNRA: c.*1222C>T	CC	CT	TT	CC+CT/TT		C	T	
Patients	31	26	10	0.028	3.33 (95% CI: 1.05-10.24)	88	46	0.22
Controls	49	46	5			144	56	
Endothelin receptor type B								
EDNRB: c.831A>G	AA	AG	GG	AA+AG/GG		A	G	
Patients	24	30	13	0.46		78	56	0.76
Controls	28	57	15			113	87	

Distribution of the genotypes and alleles of endothelin-1 (*EDN1*), endothelin receptor type A (*EDNRA*), and endothelin receptor type B (*EDNRB*) polymorphisms in normal tension glaucoma patients and controls. The p value was obtained by χ^2 or Fisher's exact test.

on the GenBank sequence of *EDN1* (NM_001955), *EDNRA* (NM_001957), and *EDNRB* (NM_000115).

Statistical analysis: The characteristics of patients were compared with those of controls using the independent t-test or χ^2 test. Comparisons of the genotype and allele distributions between patients and controls were performed using the χ^2 test or Fisher's exact test. Associations of clinical characteristics (baseline IOP, age at diagnosis, MD, and PSD) in NTG patients with genotypes were assessed using the Mann-Whitney U test. A value of $p<0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS version 13.0 (SPSS, Chicago, IL).

RESULTS

There were no differences in age, gender, or intraocular pressure between the patients with NTG and the control group (Table 2). In all SNPs, there was no evidence to reject the Hardy-Weinberg equilibrium by the χ^2 test. No linkage disequilibrium was found among polymorphisms. Of the six polymorphisms, only *EDNRA:c.*1222C>T* showed a significant association with NTG (p=0.028, OR=3.33, 95% CI 1.05-10.24; Table 3). The AA genotype of *EDNRA:c.-231G>A* was significantly associated with the lower baseline IOP than was the GG+GA genotype group (14.0±2.8 mm Hg versus 16.2±2.3 mm Hg, p=0.047; Table 4). The TT genotype of *EDNRA:c.*1222C>T* polymorphism showed a younger age at the time of diagnosis compared to the CC+CT genotype group, but with no statistical significance (39.1±8.2 years versus 45.3±10.2 years, p=0.056). All other polymorphisms were not associated with differences in baseline IOP, age at diagnosis, MD, or PSD.

DISCUSSION

In this study, analysis of the *EDN1*, *EDNRA*, and *EDNRB* genotypes showed that the polymorphism of *EDNRA:c.*1222C>T* was significantly associated with NTG, and that the AA genotype of *EDNRA:c.-231G>A* was significantly associated with the lower baseline IOP in NTG patients.

EDN1 is a potent vasoconstrictor which is implicated in the pathogenesis of various vascular diseases [12]. Because patients with NTG have normal IOP, vascular abnormalities such as vasospasm or ischemia have been thought to play a more important role in the pathogenesis of NTG than IOP [15]. Thus, it has been suggested that EDN1 may play a role in NTG pathogenesis. There are diverse effects of EDN1 upon the pathophysiology of glaucoma. First, it reduces the blood flow to the optic nerve head, resulting in ischemia and hypoxia [16]. Retinal ganglion cell death in glaucoma is thought to be initiated following ischemia, which results from prolonged vasoconstriction or vasospasm [17]. Second, it alters the axonal transport of the optic nerve, suggesting that EDN1 may have direct effects upon optic nerve function [18]. Third, it is thought to promote astrocyte proliferation in the human optic nerve head [19]. Proliferative astrocytes disrupt axonal transport, and they inhibit axon regrowth in the glaucomatous optic nerve head [20]. Fourth, EDN1 induces formation of a nitrous substance which forms toxic compounds, resulting in retinal ganglion cell death and optic nerve damage [21,22]. The various actions of EDN1 in glaucoma pathophysiology are mediated by two receptors, EDNRA and EDNRB, although the exact function of the receptors has not yet been identified. It has been thought that the function of EDN1 is maintained under the balanced influence of two receptors [23]. Therefore, subjects with qualitatively or quantitatively altered EDNRA may have dysregulation of vascular tone, or be prone to mitogenicity or other induction compounds which might lead to the risk of glaucomatous optic neuropathy in response to various stimuli.

The functional consequences of *EDNRA:c.*1222C>T* and *EDNRA:c.-231G>A* polymorphisms are unknown, since functional studies are not yet available. However, because *EDNRA:c.*1222C>T* polymorphism is in the 3'-UTR and *EDNRA:c.-231G>A* polymorphism is in the 5'-UTR, any functional consequences might be related to the regulatory se-

Table 4.

Polymorphism	Clinical characteristics	Genotype		p value
		GG+GA	AA	
EDNRA: -231G>A	Baseline IOP (mm Hg)	16.2 ±2.3	14.0 ±2.8	0.047
	Age at diagnosis (years)	43.9 ±10.3	48.7 ±8.0	0.285
	MD (dB)	-6.64±5.59	-5.47±3.18	0.839
	PSD (dB)	8.31±4.91	9.58±5.67	0.383
		CC+CT	TT	
EDNRA: c.*1222C>T	Baseline IOP (mm Hg)	16.2 ±2.5	14.8 ±1.5	0.080
	Age at diagnosis (years)	45.3 ±10.2	39.1 ±8.2	0.056
	MD (dB)	-6.68±5.43	-5.74±5.51	0.493
	PSD (dB)	8.62±4.85	7.33±5.61	0.482

Associations of clinical characteristics with genotypes of endothelin receptor type A (*EDNRA*) polymorphisms in normal tension glaucoma patients. In the table, IOP indicates intraocular pressure, MD shows the mean deviation of program 30-2 of Humphrey visual field analyzer and PSD indicates the pattern standard deviation of program 30-2 of Humphrey visual field analyzer. The p values were determined using the Mann-Whitney U test.

quences of gene transcriptions and be associated with differences in the level of gene expression. Another possibility is that such polymorphisms may create novel splice sites and thus affect the function of a receptor. In addition, such a change may be associated with other functionally active, but unidentified, gene variants. The functional significance of these polymorphisms must be examined and clarified.

A previous study reported that the systemic phenotype of the *EDNRA:c.*1222C>T* polymorphism was found to be associated with pulse pressure, the difference between systolic and diastolic blood pressure measurements [12]. Although not affecting the pathophysiology of glaucoma directly, aberrations in pulse pressure might be associated with an alteration in ocular blood flow. In this study, we did not measure the plasma EDN1 concentration. However, the EDN1 plasma concentration may not reflect vascular production, because EDN1 secretion by endothelial cells is mainly of a paracrine nature [22]. In addition, endothelin concentration in plasma in patients with NTG has been controversial [24,25].

In this study, the TT genotype of the *EDNRA:c.*1222C>T* polymorphism showed a younger age at the time of diagnosis compared to the CC+CT genotype group, although there was no statistical significance. The genetic component is more important in younger patients than in the older ones. If one has genetic predisposition to a disease, it is likely that the disease will develop earlier. Thus, our findings suggest that *EDNRA:c.*1222C>T* polymorphism might be one of the genetic risk factors for NTG in the Korean population. One might argue that there is no way to know when the disease was actually developed. However, there were no differences in the MD or PSD of the visual fields between the patients who were diagnosed at greater than or equal to 40 years of age versus those less than 40 years (data not shown). Therefore, it seems less likely that younger patients were detected earlier in the progress of the disease than were the older ones. We might also expect that this polymorphism may be associated with the patients with a family history of the condition. Although a family history of NTG is as high as 20.9% in the patients in this study, we can not show the results due to the small number of patients.

A previous study by Ishihara et al. [11] reported that the genotype distribution of *EDNRA:c.*1222C>T* polymorphism was not significantly different between open-angle glaucoma patients and normal control subjects in a Japanese population. However, open-angle glaucoma patients in that study included those with primary open-angle glaucoma (POAG) as well as NTG. In addition, that study showed that the frequency of the CT+TT genotype was higher than in control subjects in *EDNRA:c.*1222C>T* at first (p=0.036) as our study presented. However, control subjects in that study were much older than glaucoma patients by more than 10 years. Although they performed logistic regression analysis, there might be some selection bias and they were not able to show any significance after adjusting age. These findings may explain the negative results of the previous study. Since the genetic component and the pathophysiology might differ between these two conditions, POAG and NTG, we confined our study to NTG patients. In addition, racial differences might play a role in causing the different results, although both Korean and Japanese races have a high prevalence of NTG.

In this study, we have shown that the genotype of the *EDNRA* gene polymorphism was associated with a lower baseline IOP. Because non-IOP components, such as genetic factors or vascular abnormality, may play a more important role in the pathogenesis of NTG patients with lower baseline IOP, these results support the concept that EDNRA polymorphism is related to the genetic risk factor of NTG, and that it is associated with the pathophysiology of NTG.

A previous study reported that the *EDNRA:c.*70C>G* polymorphism was associated with more severe visual field defects in Japanese NTG patients [11]. We did not find any association with regard to the visual field defect. In contrast to the previous study, which used both Goldmann perimetry and Humphrey automated perimetry, and which used the subjective visual field severity scale, we performed only the Humphrey automated perimetry and thereby obtained objective MD and PSD values. We also performed the association analysis by use of a severity scale as was done in the previous study [11], but we did not find polymorphism which was associated with a worse visual field defect (data not shown).

It remains possible that some patients in the control group could eventually develop NTG, since their average age was 49 years. However, considering the overall prevalence of NTG, about 2 percent of the Korean population, it is not likely that age would have affected our results significantly [4].

In conclusion, we have shown that the polymorphism of EDNRA was associated with NTG and that it was also associated with a low baseline IOP in our Korean population. Further studies will be needed to reveal the functional influence of polymorphisms of EDNRA on gene expression and/or structure.

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