·Investigation·

Clinical relevance of the glucocorticoid receptor gene polymorphisms in glucocorticoid -induced ocular hypertension and primary open angle glaucoma

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Abstract

• AIM: To avoid the side effects of ocular hypertension of glucocorticoid (GC) usage in eye, we must identify susceptible individuals, which exists in about one-third of all population. Further, the majority of all primary open angle glaucoma (POAG) patients show this phenotype. Glucocorticoid receptor (GR) regulates C responsiveness in trabecular meshwork (TM) cells. In this study, single nucleotide polymorphism (SNP) genotyping was used to determine whether there are differences in the BclI (rs41423247) and N363S (rs6195) polymorphisms of the GR gene in healthy and POAG patients, and glucocorticoid -induced ocular hypertension (GIOH) populations.

• METHODS: Three hundred and twenty-seven unrelated Chinese adults, including 111 normal controls, 117 GIOH subjects and 99 POAG patients, were recruited. DNA samples were prepared and the BclI and N363S polymorphisms were screened using real -time polymerase chain reaction (RT-PCR)-restriction fragment length polymorphism (RFLP) analysis. Frequencies of the BclI and N363S polymorphisms were determined and compared using Fisher´s exact test and the Chi-squared test.

• RESULTS: Only the BclI polymorphism was identified in the Chinese Han population. The frequency of the G allele was 21.6 % in normal controls, 18.3% in GIOH patients, and 13.64% in the POAG patients. There was no significant difference in polymorphism or allele frequency in the 3 groups. Furthermore, no N363S polymorphism was found in the study subjects.

• CONCLUSION: The BclI polymorphisms in GR gene had no association with GIOH and POAG patients, and N363S polymorphism might not exist in the Chinese Han population. Therefore, the BclI polymorphism might not be responsible for the development of GC -induced ocular hypertension or POAG.

• **KEYWORDS:** gucocorticoid receptor; polymorphism; glucocorticoid-induced ocular hypertension; primary open-angle glaucoma
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INTRODUCTION

Certain individuals develop significant ocular hypertension when treated ocularly or systemically with glucocorticoid (GC) therapy. The use of GCs could lead to ocular hypertension and secondary open angle glaucoma that is clinically similar to primary open angle glaucoma (POAG)[1]. Although only 4% to 6% of the general population showed significantly elevated intraocular pressure (IOP) and approximately 30% showed modestly elevated IOP after GC administration, these patients were at increased risk of developing POAG later in life [2]. Almost all POAG patients develop ocular hypertension during GC therapy [3]. Furthermore, sensitivity to various clinically available GCs is rather uniform within an individual person [4] and steroid responsiveness appeared to be heritable [5]. However, the molecular mechanisms underlying the increased IOP experienced by patients with glaucoma and individuals receiving GCs are not well understood.

GCs activity is mediated through binding to the intracellular GC receptor (GR) *in vivo*. Several polymorphisms in the GR gene have been reported in the normal population, and these genetic variations might influence an individual's responsiveness to GCs [6]. One such single-nucleotide polymorphism (SNP), N363S (rs6195) in exon 2 of the GR gene (AAT AGT) was initially identified in a study of Dutch individuals who presented with hypercortisolism [7,8]. Another BclI polymorphism (rs41423247) was identified as an intronic C to G change at 646 nucleotides downstream of

Table 1 Baseline characteristics of the GIOH and POAG patients populations and normal IOP population

Parameters	Normal IOP (*n*=111)	GIOH (*n*=117)	POAG (*n*=99)
Mean age (SD)	31.2 (7.4)	29.3 (10.6)	40.5 (15.2)
Gender (% Female)	57.6	47.9	48.5
Average intraocular Pressures（mm Hg, SD）	14.3 (4.6)	25.4 (7.6)	28.7 (10.7)
Accepted LASIK operation	Y	Y	N
GC eyedrops duration	4wk	3d to 4wk	N
Anti-glaucoma drugs (%)	0	53	87
Anti-glaucoma sugery (%)	0	0	30.1

GIOH: GC-induced ocular hypertension.

exon 2 [9]. These two polymorphisms in the GR gene have been reported to be associated with increased sensitivity to GCs and might contribute to individual differences [10,11]. We hypothesize that N363S and BclI polymorphisms might play a role in GC hypersensitivity in GC-induced ocular hypertension (GIOH) or POAG.

In this study, we investigated whether the two polymorphisms (BclI and N363S) in the GR gene are involved in steroid responsiveness and/or POAG. SNP genotyping has been successfully used to identify several risk alleles and disease-associated genes [12,13]. We use this technology to determine the potential involvement of common SNPs in the human GR gene in the development of GIOH and POAG.

SUBJECTS AND METHODS

Registration number of the trial: ChiCTR-OCC-11001667, the name of the trial registry: clinical relevance of the glucocorticoid receptor gene polymorphisms with corticosteroid susceptivity in GIOH and POAG.

Subjects The protocol of this study was approved by the Ethical and Protocol Review Committee of the Third Military Medical University (Chongqing, China) and in accordance with the Declaration of Helsinki. Informed consent was obtained from each participants. A total of 327 unrelated Han Chinese adults were recruited from the Ophthalmology Department of Daping Hospital at the Third Military Medical University (Chongqing, China). Participants in the study included 168 women and 159 men (111 healthy patients, 117 GIOH patients, and 99 POAG patients) and were all from the Han ethnicity (Table 1).

The average age was 35y (range 18-59y). Patients with a history of diabetes mellitus, systemic hypertension, abnormal results in thyroid function tests, depression, or a history of having taken corticosteroid medication in the previous 3mo were excluded.

Patients were considered to have POAG (n=99) regardless of IOP if open iridocorneal angles were performed and evidence of glaucomatous optic nerve damage in at least one eye. Those with evidence of a secondary etiology of glaucoma such as pigment dispersion, pseudoexfoliation, inflammation, or a history of glucocorticoid therapy were excluded. Glaucomatous optic nerve damage was based on both optic nerve head and visual field examination. Glaucomatous optic nerve head had cup-to-disc ratios of greater than 0.7 with thinning of the neural rim, asymmetry of the optic nerve cup-to-disc ratio of >0.2, or photographic documentation of progressive loss of the neural rim. Patients were required to have visual fields of adequate quality for interpretation. For Humphrey visual fields, this required a false positive rate, false negative rate, and fixation loss rate of less than 33% [14].

The following two groups (GIOH patients and healthy normal controls) all underwent LASIK (at the Ophthalmology Department of Daping Hospital in the Third Military Medical University) and received postoperative topical steroids. The mean preoperative IOP was 16.8 mm Hg (range 10-21 mm Hg). The following selection criteria were used: 1) preoperative IOP less than or equal to 21 mm Hg measured by Goldmann applanation tonometry; 2) no personal or family history of glaucoma; 3) at least five follow-up measurements of IOP, once every week for four weeks, the last one between 2±1mo after the onset of steroid therapy; and 4) continuous steroid administration until the last studied IOP measurement or IOP elevation more than 21 mm Hg.

GIOH patients (n=117) exhibited an elevated IOP of more than 21 mm Hg after using glucocorticoid steroids eyedrops (0.1% dexamethasone), which was used for at least 4wk. During the administration of GC eyedrops, the patients' IOP was monitored every week. When the patients' IOP had risen to more than 21 mm Hg, the GC eyedrops were immediately stopped and pressure-lowering therapy was started by administering timolol maleate (0.5%), if necessary. Therefore, most of these patients did not exhibit glaucomatous optic nerve damage and visual field loss (as defined above).

The normal healthy controls (n=111) all accepted LASIK operation and glucocorticoid steroids eyedrops (0.1% dexamethasone) administration for 4wk and their IOP elevation was less than 5 mm Hg. All patients had normal slit lamp check and optic nerve head examination.

MATERIALS AND METHODS

DNA Isolation and Genotyping Genomic DNA was extracted from peripheral whole blood using the

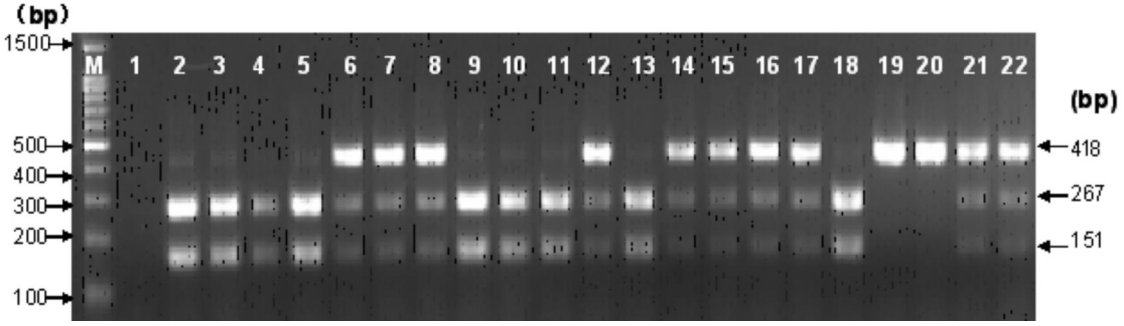

Figure 1 Genotyping of gene polymorphisms BclI: Lanes 2-5, 9-11, 13 and 18 indicate the CC genotype (151 bp and 267 bp); lanes 6-8, 12, 14-17, 21 and 22 indicate the CG genotype (151 bp, 267 bp and 418 bp); lane 19 and 20 indicates the GG genotype (only a 418 bp band).

phenol/chloroform method. The GR gene N363S and BclI polymorphisms were examined using a restriction fragment length polymorphism (RFLP) technique following polymerase chain reaction (PCR) (Mastercycler Gradient, Eppendorf, Germany). The amplification primers used for the A1220G SNP (N363S) in exon 2 of NR3C1 were AGT ACC TCT GGA GGA CAG AT (forward) and GTC CAT TCT TAA GAA ACA GG (reverse). The primers used to amplify the intron 2 of the BclI SNP were AGA GCC CTA TTC TTC AAA CTG (forward) and GAG AAA TTC ACC CCT ACC AAC (reverse)

The PCR conditions were as follows: an initial denaturation at 94℃ for 5min followed by 38 cycles of 30s at 94℃ for denaturation, 35s (BclI at 62℃ and N363S at 54℃ for annealing and 35s at 72℃ for extension, followed by a final extension for 7 min at 72℃.

Amplification of the N363S polymorphism yields a 249-bp fragment that contains two Tsp509I restriction sites which yields 3 fragments (yielding 135 + 95 + 19 bp fragments) for the wild type A-allele (N363) and only one (yielding 154 + 95 bp fragments) for the mutant G-allele (363S). Amplification of the BclI polymorphism yields a 418-bp fragment that contains a unique BclI restriction site (yielding 267 + 151 bp fragments) for the wild type C-allele and only one 418 bp fragment for the mutant G-allele. Following enzymatic digestion, PCR products were resolved on 2% agarose gel electrophoresis, visualized by ethidium bromide staining under ultraviolet light, and photographed using a video camera.

Statistical Analysis Allele frequencies for each SNP were determined by gene counting. The genotype distribution was tested for departure from the Hardy-Weinberg equilibrium using Chi-squared test. In cases of a consistent trend reflecting an allele dose effect, a linear or logistic regression analysis was performed to quantify the association. In case of a dominant or recessive effect of the test allele, analyses of variance and covariance (ANOVA and ANCOVA) tests were carried out. For dominant effects, test-allele carriers versus non-carriers were compared, whereas for recessive effects, participants homozygous for the test allele were compared with heterozygous carriers and non-carriers. The genotypic associations with the rates of GIOH and POAG occurrence were determined using the Chi-square test. The general linear model and logistic regression analyses were used to correct known confounding variables, such as age and gender ratio. The results were considered significant if P value was found to be < 0.05. All statistical analysis was performed using SPSS software version 11.0 (Chicago, IL, USA).

RESULTS

No deviation from Hardy-Weinberg equilibrium ($P1$=0.0933, $P2$=0.3755, $P3$=0.48) was seen for either polymorphism in the patients or the healthy controls. Strong linkage disequilibrium (LD) (r^2=1) was seen for the polymorphisms studied in both the patient and healthy control groups. No significant difference was found in allelic frequencies or genotype distributions based on age, gender, or group.

The N363S polymorphism was not identified in our study subjects (patient and healthy control subjects) as determined by restriction digestion of exon 2 PCR fragments with Tsp509I. The wild type GR gene showed three distinct bands of 95 bp, 135 bp and 19 bp due to the presence of two Tsp509I sites, while N363S sequence polymorphism produced two bands of 95 bp and 154 bp in the homozygous condition. The heterozygous condition showed four distinct bands of 95 bp, 135 bp, 154 bp, and 19 bp. Both patients' and control's samples showed three bands of 95 bp, 135 bp and 19 bp products (Figure 1), which confirmed the absence of the N363S polymorphism (Table 2).

The BclI polymorphism (C-G) in the GR gene was identified in all groups. The wild type genotype showed two bands of 151 bp and 267 bp while BclI polymorphism showed one band of 418 bp more in the homozygous condition, and three distinct bands of 418 bp, 267 bp and 151 bp in the heterozygous condition. Six individuals (4.88%) were found homozygous for BclI polymorphism in the GIOH group while no homozygous were found in the other two groups. Individuals who were heterozygous for BclI polymorphisms were found in all three groups: 48 individuals (43.24%) in the healthy controls, 33 individuals (26.83%) in the GIOH group,

Table 2 Genotype and allele frequency of the BclI polymorphism *n* (%)

Group	Sample size	Number of carriers of each genotype			Allelic frequency	
		CC	CG	GG	C	G
Normal IOP	111	63 (56.76)	48 (43.24)	0 (0)	78.38	21.62
GIOH	117	87 (68.29)	33 (26.83)	6 (4.88)	81.71	18.29
POAG	99	72 (72.73)	27 (27.27)	0 (0)	86.36	13.64
Total	327	213 (65.14)	108 (33.03)	6 (1.83)	80.50	19.54

and 27 individuals (27.27%) in the POAG group. The allelic frequency of the variant allele was 21.62% in the normal controls, 18.29% in GIOH group, and 13.64% in POAG group. Genotype distributions did not differ from those expected under Hardy-Weinberg equilibrium conditions. However, this observation remains uncertain since no homozygous BclI carriers and no polymorphism for N363S were found.

DISCUSSION

Single nucleotide polymorphism are variations in the DNA sequence at a frequency of greater than 1% in normal population. Studies have shown that several polymorphisms in the GR gene significantly correlated with the variability in sensitivity to endogenous glucocorticoid (GC) in normal individuals. The N363S and BclI polymorphisms are both associated with hypersensitivity to GCs in epidemiological studies [15-20]. It has been observed that GC administration can increase IOP in the general population, a condition that mimics POAG [1]. This phenomenon is especially seen in patients with POAG. The molecular mechanisms for increased steroid responsiveness are unknown. We postulated that these polymorphisms (N363S and BclI) may play a role in hypersensitivity to GCs in GIOH or POAG patients.

In the present study, we investigated the genotype and the allelic frequencies of N363S and BclI polymorphisms among GIOH, POAG patients, and healthy normal controls. No N363S polymorphism was found among these groups, as has been reported previously in a Chinese Han [15] and a Japanese population [17]. Duan *et al* [15] found no N363S polymorphism in 266 healthy volunteers and 95 trauma victims. However this finding was in contrast to the report by Huizenga *et al* [16] and Lin *et al* [17]. Huizenga reported that the frequency of the N363S polymorphism was 6.0% in a population of 216 individuals from the Netherlands, and Lin *et al* [17] reported 4% in healthy subjects from Australia. This observation may be attributed to different populations being tested, Chinese and Caucasian. However, upon further analysis, we found no significant differences in the allelic frequencies of BclI polymorphism (rs1042522) among these groups.

Many studies have shown that BclI polymorphism could induce GC hypersensitivity [18-21]. Most POAG patients are "steroid responders" or display hypersensitivity to GC induced ocular hypertension. POAG patients have also been reported to have greater sensitivity to cutaneous GC induced vasoconstriction [22]. Therefore, we determined whether Bc1I polymorphisms contributed to GIOH or POAG as a GC-sensitive related genetic factor. We classified the subjects into 3 groups (healthy normal controls, GIOH, and POAG) based on IOP and visual field loss to evaluate the changes in allelic and genotypic frequency. Even though BclI polymorphisms were observed in the Chinese Han population, the distribution of the genotypes did not differ from that expected based on Hardy-Weinberg equilibrium conditions. Our findings show that the occurrence of BclI polymorphism in intron 2 of the GR gene has no association with increased sensitivity to GC.

Our findings are consistent with recent observations by Gerzenstein *et al* [23] wherein they examined GR polymorphisms in patients who have received intravitreal triamcinolone injections. They observed no statistically significant association between any of the 6 tested GR polymorphisms and the magnitude of IOP elevation in POAG patients or steroid-responders. Among the 6 GR polymorphisms tested by the authors, these included the N363R and the BclI SNPs. Our results support these previous findings that N363S and BclI SNPs are likely not responsible for elevated IOP in response to GCs. Fingert *et al* [24] also recently reported that 22 tested GR polymorphisms have no association with GC responsiveness among GC- responders and POAG patients. It should be noted that these two studies differ from ours in several ways. In our study, we compared the polymorphisms and allelic frequency change among GC hypersensitive subjects (GIOH and POAG) and GC non-responders (controls showing normal IOP after GC use; allows for easier detection of a correlation among subject groups). Unfortunately, our SNP screening was not useful in predicting GIOH or POAG, at least in the population studied.

A variety of genetic factors may contribute to GC hypersensitivity in GIOH and POAG, or they may act in synergy with other factors. However, it remains unclear how BclI polymorphism contribute to GC hypersensitivity. Our studies are also performed in a small population. In addition, it is also known that the general frequency of polymorphisms varies greatly between ethnic populations [18]. Thus, results from one population did not necessarily apply to others. For example, the N363S polymorphism have been reported in Australia with an allele frequency of 7.4%[17], whereas in two Asian studies [15,16], no N363S carrier was found. Further, the effects of polymorphisms might differ between races due to

combinations of the different polymorphisms on several genes. Differences in environmental factors may also play an important role, making association studies performed in non-homogeneous populations difficult to interpret.

Our study tested the contribution of two GR SNPs to GC hypersensitivity in GIOH and POAG patients. Our findings show that neither N363R nor BclI SNPs are likely genetic factors influencing GC response in Han Chinese population. The etiology of steroid induced elevated IOP remains unclear, though this study provides evidence that the two common polymorphic DNA sequences in human GR are not likely genetic factors.

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