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Original Article

Nitric oxide synthase 2 gene polymorphisms are associated with prostatic volume in Korean men with benign prostatic hyperplasia

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Abstract

The precise aetiology of benign prostatic hyperplasia (BPH) remains unclear; however, it is known that immunological inflammatory processes have a role in the pathogenesis of BPH initiation and progression. Nitric oxide synthase 2 (NOS2) inducible expression is closely correlated with prostatic disease, including prostate cancer and BPH. The aim of this study was to investigate the relationship between *NOS2* polymorphisms and BPH. With a cohort of 205 controls and 229 BPH subjects, we genotyped three single nucleotide polymorphisms (SNPs) in the *NOS2* gene, including rs2779248 (promoter, –278 T/C), rs10459953 (5′-untranslated region) and rs2297518 (exon 16, missense, Ser608Leu), using direct sequencing and restriction fragment length polymorphism. The genotypic and allelic frequencies between control and BPH subjects were compared, and the associations among the BPH subjects were analyzed. SNPStats, SNPAnalyzer and HelixTree programmes were used to analyze SNPs. There was no association on SNPs between control and BPH subjects. When BPH subjects were analyzed, there was no association on SNPs between the low and high prostate-specific antigen groups. However, one SNP (rs10459953, odds ratio [OR] = 0.44, 95% confidence interval [CI] = 0.29–0.65, $P < 0.0001$, in codominant model; OR = 0.23, 95% CI = 0.12–0.46, $P < 0.0001$, in dominant model; and OR = 0.46, 95% CI = 0.24–0.86, $P = 0.015$, in recessive model) was associated with prostatic volume in BPH. We detected a strong association in genotype frequencies of *NOS2* SNP (rs10459953) between subjects with small and large prostatic volume in BPH. The result suggests that *NOS2* may be associated with prostatic volume in BPH.

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1 Introduction

Benign prostatic hyperplasia (BPH) is a pathological process that contributes to lower urinary tract symptoms (LUTS) in older men. The prevalence of BPH in Western countries is estimated at 40%–70% and in the Korean population at ~40% [1, 2]. Recent study strongly suggests that BPH is an autoimmune inflammatory disease [3]. In most prostatic diseases, immunological processes and inflammation have a role in the pathogenesis of disease initiation and/or progression. Immune inflammation-induced tissue alterations may accompany chronic processes of repetitive wound healing. Experimental investigations of prostatitis in mice and rats suggest that it is caused by an autoimmune response, with genetics as a contributing factor [4–6]. Inflammation-related molecules, such as interleukins (ILs), are associated with the development of BPH, and

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this association influences the therapeutic response of patients [7].

Nitric oxide (NO) is synthesized in various tissues through the conversion of *L*-arginine into *L*-citrulline [8]. The enzyme catalysing this reaction is designated as NO synthase (NOS) and exists in various forms: neuronal NOS (nNOS), produced by the gene nitric oxide synthase 1 (*NOS1*); endothelial NOS (eNOS), produced by the gene nitric oxide synthase 3 (*NOS3*); and inducible NOS (iNOS), produced by the gene nitric oxide synthase 2 (*NOS2*) [9–11]. The iNOS form is expressed in a number of cell types after stimulation by immune and inflammatory cytokines, as well as free radicals generated in the early stages of apoptosis and necrosis.

The expression of iNOS has been investigated in prostatic diseases, such as prostate cancer, prostatic intraepithelial neoplasia and BPH [12–14]. It is not detected in normal prostatic tissue; however, it is expressed in most BPH tissue, even in the absence of chronic prostatitis. It is suggested that genetic alterations of *NOS2* affect prostatic cell proliferation. The aim of the present study was to investigate the association between *NOS2* single nucleotide polymorphisms (SNPs) and BPH.

2 Materials and methods

2.1 Subjects

A total of 229 male subjects, from 434 patients examined, who visited the Kyung Hee University Medical Center between January 2002 and December 2007 for LUTS complaints were enrolled in the study. A total of 205 age-matched normal healthy controls were recruited from subjects visiting the hospital for routine health checkups. All healthy control subjects underwent screening and had a normal prostate-specific antigen (PSA) level (< 4.0 ng mL^{-1}). All patients provided informed consent for the use of their clinical data and samples, including DNA extracted from peripheral blood. The Institutional Review Board at Kyung Hee University Medical Center approved this study.

2.2 Sample processing

Clinical symptoms were quantified using the international prostate symptom score (IPSS), and the prostate size of all patients was assessed using transrectal ultrasonography (TRUS). Patients with a serum PSA level greater than 4 ng mL^{-1} underwent TRUS-guided prostate biopsy to rule out prostate cancer. Exclusion criteria for control and BPH patients were (a) prostate cancer, (b) neurogenic bladder, (c) urethral stricture, (d) acute/chronic prostatitis, (e) urinary tract infection, (f) uncontrolled diabetes mellitus, (g) previous pelvic surgery or (h) cardiovascular disease. BPH subjects were divided into low and high PSA groups (< 1.5 ng mL^{-1} *vs.* ≥ 1.5 ng mL^{-1}) and small and large prostatic volume groups (< 30 mL *vs.* ≥ 30 mL) [15]. Genomic DNA was extracted from blood samples collected in EDTA using the Qiagen DNA Extraction kit (Qiagen, Tokyo, Japan).

2.3 SNP selection

We selected three *NOS2* SNPs, rs2779248 (promoter, –278 T/C), rs10459953 (5′-untranslated region) and rs2297518 (exon 16, missense, Ser608Leu), with greater than 0.3 heterozygosity among SNPs located in the exon and promoter region (http://www.ncbi.nlm.nih.gov/SNP, BUILD 130) for analysis (Figure 1).

2.4 Restriction fragment length polymorphism

Genotypes of rs10459953 were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism followed by direct sequencing. Genomic DNA was amplified using the rs10459953 primers (sense, 5′-CCTGGCAGTCACAGTCATAAAT-3′; antisense, 5′-CCTGGCAGTCACAGTCATAAAT-3′; a 320-bp product; Bioneer, Daejeon, Korea). PCR consisted of 35 cycles at 94ºC for 30 s, 58ºC for 30 s and 72ºC for 1 min, and 1 cycle at 72ºC for 7 min to terminate the reaction. A 320-bp fragment was amplified, and the PCR fragment was digested with the restriction enzyme *Sau*96I (New England Biolabs, Beverly, MA, USA) (G/G: 320 bp, C/G: 320 bp, 192 bp, 128 bp and C/C: 192 bp,

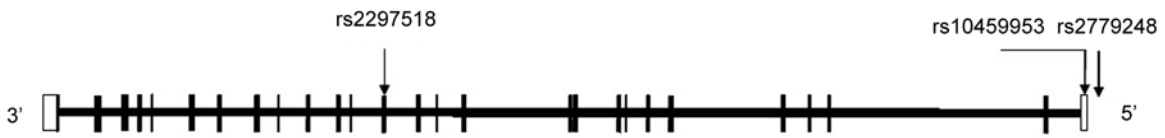

Figure 1. Gene map and single nucleotide polymorphisms (SNPs) in the *NOS2* gene. Exons are marked with boxes. The coding regions are black boxes. The first nucleotide is denoted as +1. Arrows indicate the location of each SNP.

128 bp; Figure 2). PCR products were identified using 1.5% agarose gel electrophoresis and ethidium bromide staining (Sigma, St. Louis, MO, USA).

2.5 Direct sequencing

Genotypes of rs2779248 and rs2297518 were determined through direct sequencing. Genomic DNA was amplified using the rs2779248 (sense, 5′-ACTAGTTCAGAGGCCTGTCTGG-3′; antisense, 5′-GTGACCTGATCTTGCTGTTAC-3′; a 362-bp product) and rs2297518 (sense, 5′-CAGGTAGCCCCATATGTAAACC-3′; antisense, 5′-CTGTGAGAGGGACTTGGTTTCT-3′; a 378-bp product) primers (Bioneer). PCR consisted of 35 cycles at 94ºC for 30 s, 58ºC for 30 s, 72ºC for 1 min, and 1 cycle at 72ºC for 7 min to terminate the reaction. PCR products were sequenced using an ABI PRISM 3730XL analyser (PE Applied Biosystems, Foster City, CA, USA). Sequence data were analysed using SeqManII software (v2.3; DNASTAR Inc., Madison, WI, USA).

2.6 Statistical analysis

We analyzed genetic data in control and BPH subjects. For analysis of genetic data, SNPStats (http://bioinfo.iconcologia.net/index.php), HelixTree (Golden Helix Inc., Bozeman, MT, USA) and SNPAnalyzer (ISTECH Inc., Goyang, Korea) were used. Hardy-Weinberg equilibrium (HWE) was assessed with SNPStats for both controls and cases. Multiple logistic regression models (codominant, dominant and recessive models) were used to calculate odds ratios (ORs), 95% confidence intervals (CIs) and corresponding *P*-values with Bonferroni correction while controlling for age as a covariable [16]. A linkage disequilibrium (LD) block of polymorphisms was tested using Haploview, version 4.2 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) [17]. We assessed the associations between SNPs and transition zone volume (TZV) scores using one-way analysis of variance (ANOVA) to analyze the relationship between TZV scores and genotype frequencies in the BPH group. $P < 0.05$ was considered significant.

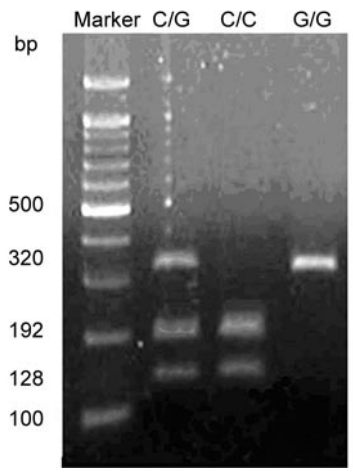

Figure 2. Representative gel displaying the polymorphic genotype of rs10459953.

3 Results

Genotype distributions of three SNPs included in this study were in HWE at all loci (rs2779248, $P = 0.44$; rs10459953, $P = 0.30$; and rs2297518, $P = 0.35$). The clinical characteristics of the 229 BPH patients are shown in Table 1. There was no difference in mean age between the BPH and control groups ($P > 0.05$). A total of 205 control and 229 BPH subjects were genotyped to investigate whether *NOS2* SNPs were associated with BPH. A representative gel displaying the polymorphic genotypes is shown in Figure 1. Genotype distributions of three SNPs for control and BPH subjects are shown in Table 2. Only rs2297518 SNP was associated with

Table 1. Baseline characteristics of 229 BPH subjects.

Characteristics	Value (mean ± SD)
Mean age (years)	65.79 ± 9.06
Prostate volume (mL)	
Total	39.15 ± 21.37
Transition zone	18.30 ± 18.87
PSA (ng mL^{-1})	
Total	3.84 ± 3.29
Free	0.98 ± 1.21
IPSS	17.64 ± 7.85
QoL	3.56 ± 1.33
Uroflowmetry	
Q_{max} (mL s^{-1})	11.29 ± 5.63
Q_{avg} (mL s^{-1})	6.45 ± 3.60
VV (mL)	208.43 ± 136.24
PVR (mL)	59.71 ± 99.43

Abbreviations: BPH, benign prostatic hyperplasia; IPSS, international prostate symptom score; PSA, prostate-specific antigen; PVR, postvoid residual volume; Q_{avg}, average flow rate; Q_{max}, maximum flow rate; QoL, quality of life; VV, voided volume.

BPH (P = 0.040 in recessive model). However, after Bonferroni correction, there was no association between control and BPH subjects (P > 0.05). *NOS2* SNPs are not associated with BPH. Table 3 shows genotype distributions of three SNPs for low and high PSA. There were no associations between PSA levels (P > 0.05). Table 4 shows genotype distributions of three SNPs in patients with low and high IPSS scores. No significant associations were found between IPSS scores (P > 0.05).

However, there were significant correlations between small prostatic volume (< 30 mL) and large prostatic volume (≥ 30 mL) groups and between specific SNPs: rs2779248 (OR = 1.99, 95% CI = 1.08–3.65, P = 0.020, in codominant model; OR = 2.11, 95% CI = 1.05–4.23, P = 0.031, in dominant model) and rs10459953 (OR = 0.44, 95% CI = 0.29–0.65, P < 0.0001, in codominant model; OR = 0.23, 95% CI = 0.12–0.46, P < 0.0001 in dominant model; OR = 0.46, 95% CI = 0.24–0.86, P = 0.015, in recessive model) (Table 5). After Bonferroni correction, rs10459953 remained with a strong correlation. The G allele of rs10459953 was found to be significantly associated with large prostatic volume and was a risk factor for increased prostatic volume in our study.

Three *NOS2* SNPs were analyzed for LD and

Table 2. Analysis of genotype frequencies in *NOS2* gene polymorphisms between control and BPH subjects.

SNP (locus)	Genotype	Group		Model	OR (95% CI)	*P*-value
		Control	BPH			
		n = 205 (%)	*n* = 229 (%)			
rs2779248	T/T	148 (72.2)	182 (79.5)	Codominant	0.79 (0.53–1.17)	0.240
Promoter	T/C	55 (26.8)	40 (17.5)	Dominant	0.66 (0.42–1.04)	0.074
–278	C/C	2 (1.0)	7 (3.1)	Recessive	2.93 (0.59–14.61)	0.160
rs10459953	G/G	69 (33.7)	66 (28.8)	Codominant	1.16 (0.88–1.52)	0.290
5'UTR	C/G	94 (45.9)	109 (47.6)	Dominant	1.23 (0.81–1.87)	0.340
	C/C	42 (20.5)	54 (23.6)	Recessive	1.21 (0.75–1.94)	0.430
rs2297518	G/G	164 (80.0)	168 (73.4)	Codominant	1.54 (0.99–2.39)	0.053
Missense	A/G	41 (20.0)	56 (24.4)	Dominant	1.47 (0.92–2.34)	0.100
Ser608Leu	A/A	0 (0.0)	5 (2.2)	Recessive		0.040*

Abbreviations: 5'UTR, 5'-untranslated region; BPH, benign prostatic hyperplasia; CI, confidence interval; *n*, number of subjects; *NOS2*, nitric oxide synthase 2; OR, odds ratio; SNP, single nucleotide polymorphism.
Note: *P*-values were obtained from logistic regression analyses with the codominant, dominant and recessive models without Bonferroni correction. *P-value was tested using Fisher's exact test.

Table 3. Analysis of genotype frequencies of *NOS2* gene polymorphisms, based on low and high PSA level, in subjects with BPH.

SNP (locus)	Genotype	PSA level (ng mL^{-1})		Model	OR (95% CI)	*P*-value
		< 1.5	≥ 1.5			
		n = 84 (%)	*n* = 144 (%)			
rs2779248	T/T	70 (83.3)	111 (77.1)	Codominant	1.26 (0.71–2.24)	0.430
Promoter	T/C	11 (13.1)	29 (20.1)	Dominant	1.50 (0.74–3.01)	0.250
–278	C/C	3 (3.6)	4 (2.8)	Recessive	0.71 (0.15–3.33)	0.670
rs10459953	G/G	23 (27.4)	43 (29.9)	Codominant	0.98 (0.68–1.44)	0.940
5'UTR	C/G	42 (50.0)	66 (45.8)	Dominant	0.88 (0.48–1.62)	0.680
	C/C	19 (22.6)	35 (24.3)	Recessive	1.10 (0.58–2.10)	0.760
rs2297518	G/G	63 (75.0)	104 (72.2)	Codominant	1.19 (0.68–2.08)	0.540
Missense	A/G	20 (23.8)	36 (25.0)	Dominant	1.16 (0.63–2.17)	0.630
Ser608Leu	A/A	1 (1.2)	4 (2.8)	Recessive	2.05 (0.22–18.92)	0.500

Abbreviations: 5'UTR, 5'-untranslated region; BPH, benign prostatic hyperplasia; CI, confidence interval; *n*, number of subjects; *NOS2*, nitric oxide synthase 2; OR, odds ratio; PSA, prostate-specific antigen; SNP, single nucleotide polymorphism.
Note: *P*-values were obtained from logistic regression analyses with the codominant, dominant and recessive models without Bonferroni correction.

Table 4. Analysis of genotype frequencies of *NOS2* gene polymorphisms, based on low and high IPPS score, in subjects with BPH.

SNP (locus)	Genotype	IPSS score		Model	OR (95% CI)	*P*-value
		0–19	20–35			
		n = 123 (%)	*n* = 88 (%)			
rs2779248	T/T	97 (78.9)	70 (79.5)	Codominant	0.97 (0.56–1.69)	0.920
Promoter	T/C	22 (17.9)	15 (17.1)	Dominant	0.96 (0.49–1.89)	0.900
–278	C/C	4 (3.2)	3 (3.4)	Recessive	1.00 (0.22–4.62)	1.000
rs10459953	G/G	33 (26.8)	31 (35.2)	Codominant	0.91 (0.62–1.33)	0.610
5'UTR	C/G	64 (52)	36 (40.9)	Dominant	0.68 (0.38–1.24)	0.210
	C/C	26 (21.1)	21 (23.9)	Recessive	1.19 (0.62–2.30)	0.610
rs2297518	G/G	86 (69.9)	70 (79.5)	Codominant	0.61 (0.34–1.11)	0.099
Missense	A/G	34 (27.6)	17 (19.3)	Dominant	0.60 (0.31–1.14)	0.110
Ser608Leu	A/A	3 (2.4)	1 (1.1)	Recessive	0.40 (0.04–3.96)	0.400

Abbreviations: 5'UTR, 5'-untranslated region; BPH, benign prostatic hyperplasia; CI, confidence interval; IPSS, international prostate symptom score; *n*, number of subjects; *NOS2*, nitric oxide synthase 2; OR, odds ratio; SNP, single nucleotide polymorphism.
Note: *P*-values were obtained from logistic regression analyses with the codominant, dominant and recessive models without Bonferroni correction.

Table 5. Analysis of genotype frequencies of *NOS2* gene polymorphisms, based on small and large prostatic volume, in subjects with BPH.

SNP (locus)	Genotype	Prostatic volume (mL)		Model	OR (95% CI)	*P*-value
		< 30 mL	≥ 30 mL			
		n = 99 (%)	*n* = 130 (%)			
rs2779248	T/T	85 (85.9)	97 (74.6)	Codominant	1.99 (1.08–3.65)	0.020
Promoter	T/C	13 (13.1)	27 (20.8)	Dominant	2.11 (1.05–4.23)	0.031
–278	C/C	1 (1.0)	6 (4.6)	Recessive	4.49 (0.53–38.38)	0.110
rs10459953	G/G	14 (14.1)	52 (40.0)	Codominant	0.44 (0.29–0.65)	< 0.0001
5'UTR	C/G	54 (54.5)	55 (42.3)	Dominant	0.23 (0.12–0.46)	< 0.0001
	C/C	31 (31.3)	23 (17.7)	Recessive	0.46 (0.24–0.86)	0.015
rs2297518	G/G	75 (75.8)	93 (71.5)	Codominant	1.40 (0.80–2.43)	0.230
Missense	A/G	24 (24.2)	32 (24.6)	Dominant	1.26 (0.69–2.31)	0.460
Ser608Leu	A/A	0 (0.0)	5 (3.8)	Recessive		0.057*

Abbreviations: 5'UTR, 5'-untranslated region; BPH, benign prostatic hyperplasia; CI, confidence interval; *n*, number of subjects; *NOS2*, nitric oxide synthase 2; OR, odds ratio; SNP, single nucleotide polymorphism.
Note: *P*-values were obtained from logistic regression analyses with the codominant, dominant and recessive models without Bonferroni correction. **P*-value was tested using Fisher's exact test.

haplotypes using Haploview (version 4.2). The LD block consisted of rs2779248 and rs10459953. Haplotypes in the LD block, however, were not associated with BPH (data not shown).

In addition, we examined the associations between *NOS2* SNPs and TZV scores. To determine the relationship between total TZV score and three SNPs, we used one-way ANOVA analysis. Of three SNPs, rs10459953 was significantly associated with TZV scores (G/G, 137.45 ± 55.48; G/C, 109.91 ± 68.92; C/C, 97.82 ± 66.48; $P = 0.002$). The patients carrying the G/G genotype had higher TZV scores than those with the C/C genotype. The G/G genotype may be correlated with an increased TZV score in patients with BPH.

4 Discussion

The present study firstly reports that rs10459953 is associated with prostatic volume in BPH subjects. The aetiology of BPH remains unclear, although evidence suggests a strong association with aging and an imbalance of sex hormones [18]. However, factors other than androgenic activity must be considered in the pathogenesis of BPH. These factors may include a number of molecules that control cellular proliferation or apoptosis, such as growth factors, hormones other than

sex hormones, cytokines and active molecules involved in immune and inflammatory responses [18]. Recently, immunological processes and inflammation have been suggested to have a role in the pathogenesis, and as potential triggers, of prostatic disease progression [3].

Konwar *et al*. [7] reported on the association between *IL4* and BPH. IL4 is a known growth stimulator for a variety of cells, while it concomitantly inhibits the release of inflammatory mediators such as tumour necrosis factor, IL6 and IL1A. They concluded that *IL4* and IL1 receptor antagonist (*ILRA*) gene polymorphisms are associated with BPH risk and influence the therapeutic response of patients. Therefore, some cytokine polymorphisms may be contributed to the risk of BPH. Lee *et al.* [19] reported about the associations among the *eNOS* G894T gene polymorphism, erectile dysfunction (ED) and BPH. Their results showed that the *eNOS* G894T polymorphism is an independent and common risk factor for both ED and BPH in men in Taiwan, China.

The generation of NO by iNOS can have cytotoxic and cytostatic effects on tumour cells, but can also promote tumour angiogenesis. The inhibition of NO has been shown to reduce blood flow in tumours, although this effect was reversed with administration of *L*-arginine. The dual role of NO in this phenomenon is presently unclear, with many conflicting findings reported in recent studies [20, 21]. The function of iNOS in the human prostate is also unclear. Klotz *et al*. [13] reported that positive iNOS immunostaining was detected in all sections of prostate cancer tissues, but that no BPH tissue stained positively for iNOS. These authors concluded that prostate cancer tissue had high iNOS content, whereas benign tissue did not. They further suggested that epithelial iNOS expression could be used as a specific immunohistochemical marker for prostate cancer. In contrast, Gradini *et al.* [14] reported that iNOS is not detected in normal prostate tissue, whereas it is expressed in all BPH tissues, even in the absence of prostatitis or systemic signs of an inflammatory condition. These authors suggested that sex hormones may be involved in iNOS induction and that there may be a role for NO in the pathogenesis in BPH. Baltaci *et al.* [12] also suggested that NO generated by iNOS may be involved in prostate cell growth, including BPH, prostatic intraepithelial neoplasia and prostate cancer. NO is closely associated with prostate carcinogenesis because of its role in cytotoxicity and angiogenesis. Lee *et al.* [19] suggested that *NOS3* gene polymorphisms represent genetic susceptibility factors for aggressive prostate cancer. However, these authors did not report an association between *NOS2* and BPH.

We suggest three mechanisms by which to explain alterations in the expression of iNOS. First, sex hormones may be linked to the induction of iNOS [22–24]. After castration of mature rats, NOS activity was reduced by 88%, 73% and 54% in the caput, corpus and cauda epididymis, respectively. These data show that androgens differentially affect NOS activity in the male reproductive tract and other organs. Second, different cytosolic concentrations of NO may alter the function of iNOS [25, 26]. A high concentration of NO, produced by iNOS, can activate enzymes such as guanylyl cyclase and transcription factors such as NF-κB. These products may be involved in controlling cell proliferation and apoptosis. The third mechanism is the effect of NO on tumour progression [27, 28]. Xie *et al*. [29] showed that the introduction of iNOS into tumour cells resulted in cytotoxicity. This finding revealed that NO is associated with tumour progression through stimulation of angiogenesis, due to the direct action of free radicals on DNA. These different mechanisms of iNOS expression by *NOS2* polymorphisms may affect prostatic enlargement.

To examine whether the genetic variants of rs2779248 interact with transcription factors, we used the online programme "AliBaba 2.1" (http://www.gene-regulation.com/pub/programs/alibaba2). At the rs2779248 site, T-containing sequences can act with Sp1 transcription factor (SP1), but SP1 disappears in C-containing sequences. These differences in transcription factor binding may influence the expression of *NOS2* directly or indirectly. The rs2297518 SNP located in exon 16 of the *NOS2* gene is a missense SNP. The SNP change affects mRNA (TCG$\rightarrow$TTG, C2087T) and protein (serine$\rightarrow$leucine, Ser608Leu) production.

The major limitation of our study was the small sample size used for comparison within the BPH group. However, we performed the first genetic examination of the relationship between *NOS2* SNPs and BPH using standard diagnostic tools. Our results revealed a strong association between *NOS2* and BPH. In the current study, the *NOS2* SNP, rs10459953, was found to be significantly associated with prostatic volume in men with BPH.

In conclusion, we observe that there is a strong association between the *NOS2* SNP, rs10459953 and prostatic volume in men with BPH. Studies on other *NOS2* SNPs and different population will be needed.

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