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Association between *VEGF* and *eNOS* gene polymorphisms and lumbar disc degeneration in a young Korean population

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ABSTRACT. Disturbances in blood flow to intervertebral discs (IVD) play an important role in IVD degeneration. Vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) are extremely important angiogenic factors for vasodilation and neovascularization. We investigated the relationship between single nucleotide polymorphisms (SNPs) of the *VEGF* and *eNOS* genes and genetic susceptibility to lumbar IVD degeneration in a young adult Korean population. Two hundred and forty-one participants (aged 18 to 30 years), with or without low back pain, were selected for the

study. Magnetic resonance imaging was made of the lumbar spine in all participants. The patient group (N = 102) had low back pain clinically and lumbar IVD degeneration radiographically. The control group (N = 139) included subjects with and without low back pain; all were negative radiographically for lumbar IVD degeneration. Using PCR-RFLP analysis, we analyzed *VEGF* (-2578C>A, -1154G>A, -634G>C, and 936C>T) and *eNOS* (-786T>C, 4a4b and 894G>T) SNPs. We made combined analyses of the genes and performed haplotype analyses. There were no significant differences in the genotype distribution of polymorphisms of *VEGF* and *eNOS* genes among patients and controls. However, the frequency of *VEGF* -2578CA +AA/-634CC combined genotypes was significantly higher in patients when compared with controls [odds ratio (OR) = 21.00; 95% confidence interval (CI) = 2.590-170.240]. The frequencies of the -2578A/-1154A/-634C/936C (OR = 3.831; 95%CI = 1.068-13.742), -2578A/-1154A/-634C (OR = 3.356; 95%CI = 1.198-9.400), and -2578A/-634C/936C (OR = 10.820; 95%CI = 2.811-41.656) haplotypes were also significantly higher in patients than in controls. We conclude that the combined genotype *VEGF* -2578CA+AA/-634CC is a possible risk factor for IVD degeneration and the *VEGF* -2578A/-1154A/-634C/936C haplotype may increase the risk for development of IVD degeneration. Furthermore, the *VEGF* -634C allele appears to be associated with susceptibility to IVD degeneration.

Key words: Endothelial nitric oxide synthase; Polymorphism; Intervertebral disc degeneration; Vascular endothelial growth factor

INTRODUCTION

The lumbar intervetebral disc (IVD) is the largest avascular tissue, and adequate nutritional supply is critical for maintenance of a healthy IVD. This supply from the surrounding vasculature occurs mainly through diffusion from the vertebral endplates with a minor contribution from the vessels surrounding the periphery of the annulus (Shi et al., 2011). Disc cells depend on the blood supply from the capillaries at their margins to supply nutrients and remove metabolic waste. Therefore, blood flow disturbance in the IVD has been proposed to play a role as a causative factor in the IVD degeneration (Nachemson et al., 1970; Buckwalter, 1995; Urban et al., 2004; Awata et al., 2005; Niinimäki et al., 2009; Shi et al., 2011). The lumbar IVD is mostly supplied by the capillaries of lumbar arteries feeding the vertebral endplates, and the blood supply can be disturbed by lumbar arteries narrowing due to atherosclerosis or by occlusion of endplate openings due to morphological changes (Kurunlahti et al., 1999, 2001; Benneker et al., 2005; Moore, 2006; Niinimäki et al., 2009; Kauppila, 2009; Wang and Griffith, 2011). Thus, atherosclerosis and other cardiovascular risk factors have received growing attention as potential underlying factors for IVD degeneration (Kauppila and Tallroth, 1993; Kurunlahti et al., 1999; Kauppila et al., 2004; Leino-Arjas et al., 2008; Kauppila, 2009). Characteristic features of IVD degeneration include nuclear dehydration, proteoglycan loss, decreased cellularity, disorganization, and annular disruption (Lotz, 2004). Another feature of IVD degeneration is blood vessel ingrowth in the degenerated disc through the vertebral endplate or through

the annulus fibrosus (Brown et al., 1997; Freemont et al., 2002; Haro et al., 2002). Since atherosclerosis is associated with IVD degeneration and since angiogenesis occurs as a degenerative process of the IVD, it is reasonable to hypothesize that genes related to atherosclerosis and angiogenesis are candidates to determine the risk of developing IVD degeneration. Therefore, single nucleotide polymorphisms (SNPs) in genes previously implicated in angiogenesis or atherosclerosis may contribute to the etiology of IVD degeneration.

With regard to atherosclerosis and angiogenesis, vascular endothelial growth factor (VEGF) and nitric oxide (NO) are important. VEGF is a key mediator of angiogenesis, and NO is a regulator of VEGF-mediated angiogenesis (La Rosa et al., 2003; Lin et al., 2010). VEGF induces NO production from vascular endothelial cells via endothelial NO synthase (eNOS; Fukumura et al., 2001). Thus, SNPs of the *VEGF* and *eNOS* genes have been shown to be associated with the development of atherosclerosis, coronary heart disease, and ischemic stroke (Lin et al., 2010; Song et al., 2010; Kim et al., 2007, 2011). Our group investigated the effect of *VEGF* -2578C>A, -1154G>A, -634G>C, and 936C>T polymorphisms on susceptibility to ischemic stroke and also investigated an association of *eNOS* (-786T>C, 4a4b, and 894G>T) polymorphisms with silent brain infarction and coronary artery disease (Song et al., 2010; Kim et al., 2007, 2011). Until now, however, no published studies explored the association of polymorphisms in *VEGF* and *eNOS* genes with genetic susceptibility to IVD degeneration. Therefore, we aimed to investigate whether SNPs in the *VEGF* and *eNOS* genes are associated with susceptibility to IVD degeneration in the Korean population.

MATERIAL AND METHODS

Study population

The case-control population comprised 241 adult unrelated Korean residents (age 18-30 years) who were selected between 2009 and 2011. A total of 102 lumbar IVD degeneration cases (68 males, 34 females; mean age ± standard deviation, 23.6 ± 6.3 years) were recruited from CHA Bundang Medical Center. All patients had clinically evident low back pain and radiographically evident lumbar IVD degeneration (grade III or higher, according to the 5-grade classification system) (Pfirrmann et al., 2001). The control group consisted of 139 healthy volunteers (80 men, 59 women; 23.4 ± 4.1 years) with no history of lumbar trauma or back problems. All controls were examined by MRI to exclude individuals with asymptomatic lumbar IVD degeneration. Lumbar spine MRI showed grade I or II discs according to the Pfirrmann grading system (Pfirrmann et al., 2001). None of the subjects were involved in heavy physical labor or were exposed to vibration at work, and all participants were non-smokers. We excluded subjects with history of stroke or ischemic heart disease, diagnosis with endocrine disorders such as hyperthyroidism, primary hyperparathyroidism, pituitary diseases, diabetes mellitus, liver disease, hypercholesterolemia, or renal disease. Patients receiving treatments with the potential to interfere with bone metabolism were also excluded, such as oral contraceptives, hormone replacement therapy, corticosteroids, calcium, or vitamin D. No subject was obese or had a body mass index >30 kg/m^2.

The study was approved by the Institutional Review Board of CHA Bundang Medical Center, and written informed consent was obtained from all participants in this study.

Grading of IVD degeneration and severity score

The lumbar spines of the participants were imaged with a 1.5-Tesla MRI unit (Signa®

HDxt 1.5T; GE Healthcare, Milwaukee, WI, USA). The presence of lumbar IVD degeneration was determined from T2-weighted sagittal images (2000-ms repetition time and 110-ms echo time). Two independent radiologists assessed the signal intensity of individual discs between the L2-L3 and L5-S1 level. The cerebrospinal fluid adjacent to the corresponding disc level was used as a reference for signal intensity. The grade of IVD degeneration was determined according to the Pfirrmann classification system (Pfirrmann et al., 2001). Grade I indicated homogeneous with a bright hyperintense signal intensity and a normal disc height; grade II, inhomogeneous with a hyperintense signal and a normal disc height; grade III, inhomogeneous with an intermediate gray signal intensity and a normal or slightly decreased disc height; grade IV, inhomogeneous with a hypointense gray signal and a normal or moderately decreased disc height; and grade V, inhomogeneous with a hypointense black signal intensity and a collapsed disc height. The presence of disc herniation was also evaluated. We included only patients with degenerative IVD grade III or higher and excluded patients with clearly herniated or extruded discs. The presence of multilevel IVD degeneration was assessed as the number of disc levels with the same degenerative change. A 'severity score' was developed to grade the global degree of IVD degeneration within the lumbar spine, with this score determined on the basis of the numbers of degenerative discs between L2-L3 and L5-S1 (e.g., 1 = one degenerated level).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the G-DEX blood extraction kit (iNtRON Biotechnology, Inc., Seongnam, South Korea). Four SNPs in the *VEGF* gene were studied, which included 2 promoter region SNPs (-2578C>A, rs699947 and -1154G>A, rs1570360), 1 leader sequence (-634G>C, rs2010963), and one 3'-untranslated region SNP (936C>T, rs3025039). All SNP sequences were obtained from the HapMap database (www.hapmap.org). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was performed to analyze the *VEGF* -2578C>A and 936C>T polymorphisms. The *VEGF* -1154G>A and -634G>C polymorphisms were analyzed by real-time PCR. The primers and PCR conditions for the determination of *VEGF* SNP have been previously described (Del Bo et al., 2005).

The 3 SNPs of the *eNOS* gene were selected, which included -786T>C, rs2070744; 894G>T, rs1799983, and *eNOS* 4a4b [27-bp variable number of tandem repeat (VNTR) polymorphism in intron 4]. PCR-RFLP assay was performed to analyze these 3 SNPs. The primers and PCR conditions for the determination of *eNOS* SNP have been previously described (Inoue et al., 1998).

Statistical analysis

Genotype frequencies were compared between cases and controls using the chi-square test and the Fisher exact test when appropriate, and odds ratios (OR) and 95% confidence intervals (CI) were calculated to estimate the relative risk of IVD degeneration conferred by each polymorphism. Association of SNPs with the IVD degeneration was analyzed by multivariate logistic regression adjusted for possible confounders, including age and gender. Demographic data between groups were compared by the chi-square test for categorical variables such as gender and by the Mann-Whitney test for quantitative variables such as age. The haplotypes were classified using the HAPSTAT 3.0 software (http://dlin.web.unc.edu/software/hapstat/), and association between *VEGF* and *eNOS* polymorphisms and IVD degeneration was estimated by OR

and 95%CI. Statistical analyses were performed using the SPSS software (version 11.0; SPSS Inc., Chicago, IL, USA) and the StatsDirect Statistical Software (Version 2.4.4, StatsDirect Ltd., Altrincham, UK). The level of statistical significance was set at $P < 0.05$.

RESULTS

There were no significant differences between cases and controls for mean age or gender distribution. Among the patients with the IVD degeneration, the disc degeneration severity score was analyzed according to the genotypes of *VEGF* and *eNOS*. No significant differences were observed in severity scores between the genotypes for each SNP in the *VEGF* and *eNOS* genes (Table 1). Therefore, we investigated the association between SNPs in *VEGF* and *eNOS* genes and patients with any severity score >1. A comparison of genotype and allele frequencies of the *VEGF* -2578C>A, -1154G>A, -634G>C, and 936C>T polymorphisms and *eNOS*-786T>C, intron 4, and 894G>T polymorphisms between case and control groups is shown in Table 2. Genotype distributions of each polymorphism did not deviate from those expected based on the Hardy-Weinberg equilibrium. The frequencies of *VEGF* -2578CA+AA (51.0%), -1154GA+AA (29.4%), -634GC+CC (74.5%), *eNOS* -786TC+CC (21.6%), and 894GT+TT (27.5%) were higher in the patients compared with controls. However, no statistically significant differences between patients and controls were found (Table 2).

Table 1. Severity score levels of *eNOS* and *VEGF* polymorphisms in patients with disc degeneration.

	Number (%)	Score (means ± SD)	P[a]
eNOS -786T>C			
TT	118 (84.9)	2.750 ± 1.268	Reference
TC	21 (15.1)	2.571 ± 1.121	0.549
CC	0 (0.0)	-	-
TC+CC	21 (15.1)	2.545 ± 1.101	0.496
eNOS 4a4b			
4b4b	115 (82.7)	2.750 ± 1.268	Reference
4a4b	24 (17.3)	2.571 ± 1.121	0.549
4a4a	0 (0.0)	-	-
4a4b+4a4a	24 (17.3)	2.545 ± 1.101	0.496
eNOS 894G>T			
GG	111 (79.9)	2.813 ± 1.193	Reference
GT	25 (18.0)	2.455 ± 1.335	0.256
TT	3 (2.1)	2.200 ± 1.304	0.288
GT+TT	28 (20.1)	2.407 ± 1.309	0.158
VEGF -2578C>A			
CC	75 (54.0)	2.800 ± 1.161	Reference
CA	55 (39.5)	2.707 ± 1.309	0.816
AA	9 (6.5)	2.273 ± 1.272	0.215
CA+AA	64 (46.0)	2.615 ± 1.301	0.520
VEGF -1154G>A			
GG	102 (73.4)	2.625 ± 1.238	Reference
GA	28 (20.1)	3.000 ± 1.243	0.206
AA	9 (6.5)	2.571 ± 1.134	0.945
GA+AA	37 (26.6)	2.900 ± 1.213	0.300
VEGF -634G>C			
GG	48 (34.5)	2.885 ± 1.211	Reference
GC	64 (46.0)	2.692 ± 1.213	0.553
CC	27 (19.5)	2.542 ± 1.318	0.374
GC+CC	91 (65.5)	2.645 ± 1.240	0.432
VEGF 936C>T			
CC	95 (68.3)	2.821 ± 1.235	Reference
CT	38 (27.4)	2.348 ± 1.191	0.124
TT	6 (4.3)	2.000	-
CT+TT	44 (31.7)	2.333 ± 1.167	0.108

eNOS = endothelial nitric oxide synthase; *VEGF* = vascular endothelial growth factor; SD = standard deviation. [a]Calculated by the Mann-Whitney U-test.

Table 2. Comparison of genotype frequencies of *eNOS* and *VEGF* polymorphisms between the patients and control subjects.

Genotypes	Control (%)	Case (%)	OR (95%CI)	P
eNOS -786T>C				
TT	118 (84.9)	80 (78.4)	1.000	
TC	21 (15.1)	21 (20.6)	1.475 (0.756-2.878)	0.253
CC	0 (0.0)	1 (1.0)	4.416 (0.178-109.856)	0.407
TC+CC	21 (15.1)	22 (21.6)	1.545 (0.797-2.996)	0.196
eNOS 4a4b				
4b4b	115 (82.7)	80 (78.4)	1.000	
4a4b	24 (17.3)	21 (20.6)	1.258 (0.656-2.413)	0.490
4a4a	0 (0.0)	1 (1.0)	4.304 (0.173-107.088)	0.413
4a4b+4a4a	24 (17.3)	22 (21.6)	1.318 (0.691-2.512)	0.401
eNOS 894G>T				
GG	111 (79.9)	74 (72.5)	1.000	
GT	25 (18.0)	23 (22.6)	1.380 (0.729-2.613)	0.322
TT	3 (2.1)	5 (4.9)	2.500 (0.580-10.782)	0.276
GT+TT	28 (20.1)	28 (27.5)	1.500 (0.823-2.736)	0.185
VEGF -2578C>A				
CC	75 (54.0)	50 (49.0)	1.000	
CA	55 (39.5)	41 (40.2)	1.118 (0.652-1.919)	0.685
AA	9 (6.5)	11 (10.8)	1.833 (0.708-4.746)	0.207
CA+AA	64 (46.0)	52 (51.0)	1.219 (0.730-2.034)	0.449
VEGF -1154G>A				
GG	102 (73.4)	72 (70.6)	1.000	
GA	28 (20.1)	23 (22.5)	1.164 (0.621-2.182)	0.636
AA	9 (6.5)	7 (6.9)	1.102 (0.392-3.096)	0.854
GA+AA	37 (26.6)	30 (29.4)	1.149 (0.651-2.028)	0.633
VEGF -634G>C				
GG	48 (34.5)	26 (25.5)	1.000	
GC	64 (46.0)	52 (51.0)	1.500 (0.822-2.737)	0.185
CC	27 (19.5)	24 (23.5)	1.641 (0.792-3.399)	0.181
GC+CC	91 (65.5)	76 (74.5)	1.542 (0.875-2.716)	0.133
VEGF 936C>T				
CC	95 (68.3)	78 (76.5)	1.000	
CT	38 (27.4)	23 (22.5)	0.737 (0.405-1.341)	0.317
TT	6 (4.3)	1 (1.0)	0.203 (0.024-1.723)	0.137
CT+TT	44 (31.7)	24 (23.5)	0.664 (0.372-1.187)	0.166

OR = odds ratio; CI = confidence interval. P values were adjusted for age and gender. For other abbreviations, see legend to Table 1.

The linkage disequilibrium (LD) for SNPs in the *VEGF* and *eNOS* genes in patients is shown in Figure 1. D prime (D') and the square of the correlation coefficient (r^2) were calculated to assess the level of LD between each SNP. There was strong LD between *eNOS* -786 and 4a4b (D' = 0.975, r^2 = 0.883) in patients (Figure 1A). Polymorphisms *VEGF* -2578C>A and -1154G>A were in moderate level of LD (D' = 0.732, r^2 = 0.284) in patients (Figure 1B).

The combination analysis of *VEGF* and *eNOS* gene polymorphisms is shown in Tables 3 and 4. The frequencies of *VEGF* -2578CA+AA/-634CC combined genotypes were significantly higher in patients with lumbar IVD degeneration compared with controls (OR = 21.000; 95%CI = 2.590-170.240; P = 0.000) (Table 3). The frequencies of *eNOS* combined genotypes were not statistically different between case and control groups (Table 4).

We constructed separate haplotypes with 4 *VEGF* and 3 *eNOS* polymorphisms to determine whether any specific haplotypes were associated with IVD degeneration (Tables 5 and 6). The frequencies of the A-A-C-C (*VEGF* -2578/-1154/-634/936; OR = 3.831; 95%CI = 1.068-13.742; P = 0.002), A-A-C (*VEGF* -2578/-1154/-634; OR = 3.356; 95%CI = 1.198-9.400; P = 0.001), and A-C-C (*VEGF* -2578/-634/-936; OR = 10.820; 95%CI = 2.811-41.656; P < 0.0001) haplotypes were also significantly higher in patients

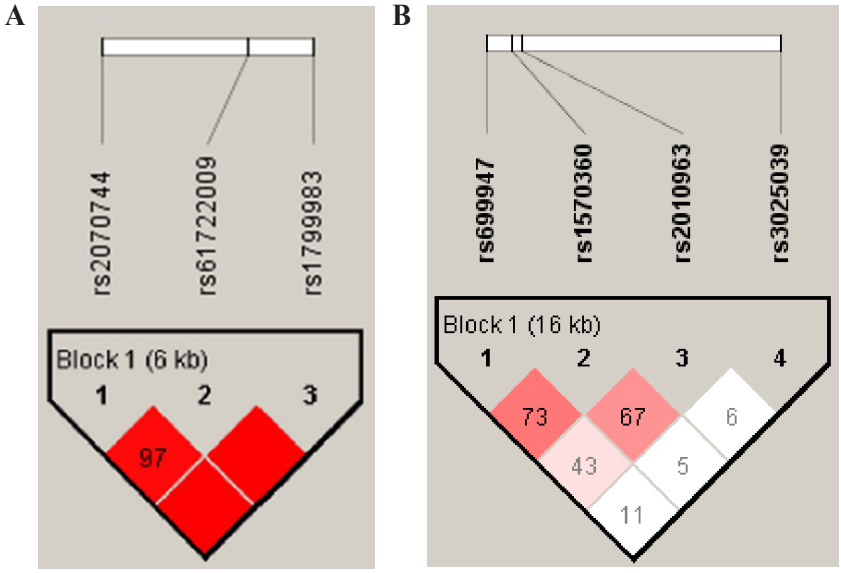

Figure 1. A. Linkage disequilibrium (LD) analysis of single nucleotide polymorphisms (SNPs) [-786 (rs2070744), 27-bp VNTR, and +894 (rs1799983)] in the *eNOS* gene. Values in squares are LD between single markers. **B.** LD analysis of SNPs [-2578 (rs699947), -1154 (rs1570360), -634 (rs2010963), and +936 (rs3025039)] in the *VEGF* gene.

Table 3. Combination analysis of *VEGF* polymorphisms in patients and controls.

Genotypes	Control (%)	Case (%)	OR (95%CI)	P
VEGF -2578C>A /-1154G>A				
CC/GG	74 (53.2)	45 (44.1)	1.000	
CC/GA+AA	1 (0.7)	5 (4.9)	8.222 (0.930-72.686)	0.037
CA+AA/GG	28 (20.1)	27 (26.5)	1.586 (0.831-3.025)	0.160
CA+AA/GA+AA	36 (25.9)	25 (24.5)	1.142 (0.608-2.146)	0.680
VEGF -2578C>A /-634G>C				
CC/GG+GC	49 (35.3)	38 (37.3)	1.000	
CC/CC	26 (18.7)	12 (11.8)	0.808 (0.353-1.847)	0.612
CA+AA/GC+CC	63 (45.3)	40 (39.2)	1.111 (0.603-2.046)	0.735
CA+AA/CC	1 (0.7)	12 (11.8)	21.000 (2.590-170.240)	0.000
VEGF -2578C>A /936C>T				
CC/CC	54 (38.8)	39 (38.2)	1.000	
CC/CT+TT	21 (15.1)	11 (10.8)	0.725 (0.314-1.677)	0.451
CA+AA/CC	41 (29.5)	39 (38.2)	1.317 (0.722-2.404)	0.369
CA+AA/CT+TT	23 (16.5)	13 (12.7)	0.783 (0.353-1.734)	0.545
VEGF -1154G>A /-634G>C				
GG/GG+GC	76 (54.7)	52 (51.0)	1.000	
GG/CC	26 (18.7)	20 (19.6)	1.124 (0.569-2.223)	0.736
GA+AA/GG+GC	36 (25.9)	26 (25.5)	1.056 (0.570-1.954)	0.863
GA+AA/CC	1 (0.7)	4 (3.9)	5.846 (0.635-53.831)	0.161
VEGF -1154G>A /936C>T				
GG/CC	72 (51.8)	57 (55.9)	1.000	
GG/CT+TT	30 (21.6)	15 (14.7)	0.632 (0.310-1.286)	0.203
GA+AA/CC	23 (16.5)	21 (20.6)	1.153 (0.581-2.291)	0.684
GA+AA/CT+TT	14 (10.1)	9 (8.8)	0.812 (0.328-2.011)	0.652
VEGF -634G>C / 936C>T				
GG+GC/CC	78 (56.1)	63 (61.8)	1.000	
GG+GC/CT+TT	34 (24.5)	15 (14.7)	0.546 (0.273-1.092)	0.085
CC/CC	17 (12.2)	15 (14.7)	1.092 (0.506-2.359)	0.822
CC/CT+TT	10 (7.2)	9 (8.8)	1.114 (0.427-2.910)	0.825

P values were adjusted for age and gender. For abbreviations, see legends to Tables 1 and 2.

Table 4. Combination analysis of *eNOS* polymorphisms in patients and control subjects.

Genotypes	Control (%)	Case (%)	OR (95%CI)	P
eNOS -786T>C/4a4b				
TT/4b4b	115 (82.7)	79 (77.4)	1.000	
TT/4a4b	3 (2.2)	1 (1.0)	0.485 (0.050-4.752)	0.649
TC/4b4b	0 (0.0)	1 (1.0)	4.359 (0.175-108.446)	0.410
TC/4a4b	21 (15.1)	20 (19.6)	1.386 (0.705-2.726)	0.342
CC/4a4a	0 (0.0)	1 (1.0)	4.359 (0.175-108.446)	0.410
eNOS -786T>C/894G>T				
TT/GG	90 (64.7)	54 (52.9)	1.000	
TT/GT	25 (18.0)	21 (20.6)	1.400 (0.716-2.739)	0.325
TT/TT	3 (2.2)	5 (4.9)	2.778 (0.638-12.093)	0.262
TC/GG	21 (15.1)	20 (19.6)	1.587 (0.789-3.194)	0.193
TC/GT	0 (0.0)	1 (1.0)	4.982 (0.199-124.557)	0.379
CC/GG	0 (0.0)	1 (1.0)	4.982 (0.199-124.557)	0.379
eNOS 4a4b/894G>T				
4b4b/GG	87 (62.5)	53 (51.9)	1.000	
4b4b/GT	25 (18.0)	22 (21.6)	1.445 (0.741-2.815)	0.279
4b4b/TT	3 (2.2)	5 (4.9)	2.736 (0.628-11.921)	0.263
4a4b/GG	24 (17.3)	21 (20.6)	1.436 (0.729-2.830)	0.294
4a4a/GG	0 (0.0)	1 (1.0)	4.907 (0.196-122.732)	0.383
eNOS -786T>C/4a4b/894G>T				
TT/4b4b/GG	87 (62.5)	53 (51.9)	1.000	
TT/4b4b/GT	25 (18.0)	21 (20.6)	1.379 (0.703-2.704)	0.349
TT/4b4b/TT	3 (2.2)	5 (4.9)	2.736 (0.628-11.921)	0.263
TT/4a4b/GG	3 (2.2)	1 (1.0)	0.547 (0.055-5.400)	1.000
TC/4b4b/GT	0 (0.0)	1 (1.0)	4.907 (0.196-122.732)	0.38
TC/4a4b/GG	21 (15.1)	20 (19.6)	1.563 (0.775-3.152)	0.210
CC/4a4a/GG	0 (0.0)	1 (1.0)	4.907 (0.196-122.732)	0.383

P values were adjusted for age and gender. For abbreviations, see legends to Tables 1 and 2.

than in controls (Table 5). There were no significant differences between patient and control groups for haplotypes constructed from *eNOS* SNP (Table 6).

DISCUSSION

We conducted a case-control study to investigate the relationship between SNPs of the *VEGF* and *eNOS* genes, their haplotypes, and IVD degeneration in a young Korean population. The data suggested that the combined genotypes of *VEGF* -2578CA+AA/-634CC were significantly associated with IVD degeneration. In addition, -2578A/-1154A/-634C/936C, -2578A/-1154G/-634C, and -2578A/-634C/936C haplotypes were associated with increased risk of IVD degeneration, whereas -2578A/-1154A/-634G and -2578A/-1154G/-634G were protective against IVD degeneration. The *VEGF* haplotypes associated with high risk of IVD degeneration contain the -634C allele. Thus, the *VEGF* -634C allele may be important for susceptibility to IVD degeneration.

The effects of polymorphisms in the *VEGF* gene are likely to be manifested as differential expression of VEGF protein between individuals and may be linked to a difference in susceptibility to IVD degeneration. Clinical studies have shown that the *VEGF* SNP -2578A and -1154A alleles decrease VEGF expression, but *VEGF* -634G>C SNP has been correlated with a weak ability for VEGF production (Watson et al., 2000; Awata et al., 2002; Niinimäki et al., 2009). Awata et al. (2005) proposed that the VEGF -634C allele is associated with increased transcription levels of VEGF, whereas Lambrechts et al. (2005) reported that VEGF -634G is associated with lower VEGF expression. However, our study was limited in this respect, since we did not measure VEGF levels to show the association between them and the development of IVD degeneration.

Table 5. Haplotype analysis of *VEGF* polymorphisms in patients and controls.

Haplotypes	Control (%)	Case (%)	P	OR (95%CI)
VEGF -2578C>A /-1154G>A/-634G>C/936C>T				
C-G-C-C	34.2	25.3	0.036	0.842 (0.722-0.982)
C-G-G-C	26.0	32.1	0.116	1.150 (0.959-1.379)
A-A-G-C	9.5	5.4	0.084	0.797 (0.641-0.992)
A-G-G-C	9.3	4.8	0.068	0.784 (0.630-0.975)
C-G-C-T	7.2	4.3	0.208	0.827 (0.640-1.070)
C-G-G-T	4.0	2.6	0.366	0.835 (0.594-1.172)
A-A-G-T	3.7	1.2	0.069	0.685 (0.526-0.893)
A-G-G-T	2.8	0.0	0.015	0.571 (0.528-0.617)
C-A-G-C	2.4	4.1	0.377	1.245 (0.721-2.151)
A-A-C-C	0.7	5.6	0.002	3.831 (1.068-13.742)
A-A-C-T	0.3	1.2	0.390	1.738 (0.350-8.628)
A-G-C-C	0.0	10.4	<0.0001	-
A-G-C-T	0.0	2.3	0.009	-
C-A-G-T	0.0	0.7	0.242	-
VEGF -2578C>A/-1154G>A/-634G>C				
C-G-C	41.5	29.5	0.006	0.806 (0.694-0.936)
A-A-G	13.2	6.6	0.014	0.755 (0.628-0.908)
A-G-G	12.1	4.7	0.006	0.722 (0.603-0.865)
A-A-C	1.0	6.7	0.001	3.356 (1.198-9.400)
A-G-C	0.0	12.9	<0.0001	
VEGF -2578C>A /-1154G>A/936C>T				
C-G-T	11.2	7.1	0.165	0.842 (0.678-1.046)
A-G-C	9.4	14.9	0.068	1.276 (0.953-1.709)
VEGF -2578C>A/-634G>C/936C>T				
C-C-C	34.1	25.2	0.032	0.839 (0.720-0.979)
C-G-C	28.4	36.2	0.063	1.174 (0.984-1.400)
A-G-C	18.8	10.4	0.009	0.772 (0.652-0.913)
A-G-T	6.4	1.0	0.003	0.626 (0.530-0.740)
A-C-C	0.7	16.0	<0.0001	10.820 (2.811-41.656)
A-C-T	0.3	3.6	0.009	4.682 (0.747-29.346)
VEGF -1154G>A/-634G>C/936C>T				
G-G-T	7.0	3.0	0.042	0.737 (0.588-0.923)
A-C-C	0.0	4.2	0.000	-

P values were adjusted for age and gender. For abbreviations, see legends to Tables 1 and 2.

Atherosclerosis has been found to be associated with IVD degeneration, and angiogenesis has been shown to be part of the degenerative process of IVD. However, it remains uncertain whether VEGF enhances or prevents IVD degeneration. Increased VEGF expression has been associated with the progress of atherosclerosis through neovascularization and inflammation in atheromatous plaques (Inoue et al., 1998; Celletti et al., 2001). Some studies have shown that expression of VEGF and nerve growth factor are significantly higher in degenerative disc with no herniation compared with herniated disc, and angiogenesis is related to the presence of nerve fiber ingrowth into degenerative IVD and the appearance of discogenic low back pain (Freemont et al., 1997, 2002). Additionally, VEGF has been suggested to promote ectopic calcification via increase in angiogenesis (Karamouzian et al., 2010). Thus, we can infer that increased VEGF expression may aggravate IVD degeneration through the progress of atherosclerosis or calcification leading to occlusion of endplate openings, and further exacerbate low back pain by inducing nociceptive nerve ingrowth into degenerated disc. Conversely, others suggest that the VEGF is protective against IVD degeneration via the neovascularization of the degenerative disc (Haro et al., 2002; Kato et al., 2004; Walsh, 2004). Angiogenesis may be regarded as a part of the repair response (Haro et al., 2002). VEGF may

play a role in the resorption of the herniated disc, and the tendency of the herniated disc to spontaneous resorption is directly proportional to the degree of neovascularization (Haro et al., 2002). VEGF-induced neovascularization may explain why the symptoms of sciatic pain gradually improve without surgical treatment in some patients with herniated discs (Haro et al., 2002; Zhao et al., 2009). These previous theories and data support a strong relationship between the IVD and VEGF.

Table 6. Haplotype analysis of *eNOS* polymorphisms in patients and controls.

Haplotypes	Control (%)	Case (%)	P	OR (95%CI)
eNOS -786T>C/4a4b/894G>T				
T-4b-G	80.2	73.0	0.064	0.834 (0.680-1.023)
T-4b-T	11.1	15.3	0.190	1.176 (0.906-1.528)
C-4a-G	7.6	10.7	0.219	1.199 (0.874-1.644)
T-4a-G	1.1	0.5	0.481	0.767 (0.433-1.358)
C-4b-T	0.0	0.5	0.243	-
eNOS -786T>C/4a4b				
T-4b	91.3	88.3	0.257	0.854 (0.637-1.146)
C-4a	7.6	10.7	0.219	1.199 (0.874-1.644)
T-4a	1.1	0.5	0.481	0.767 (0.433-1.358)
C-4b	0.0	0.5	0.243	-
eNOS -786T>C/894G>T				
T-G	81.2	73.9	0.056	0.826 (0.670-1.019)
T-T	11.2	15.7	0.145	1.198 (0.921-1.559)
C-G	7.6	10.4	0.292	1.168 (0.855-1.597)
eNOS 4a4b/894G>T				
4b-G	80.2	73.0	0.064	0.834 (0.680-1.023)
4b-T	11.2	15.7	0.145	1.198 (0.921-1.559)
4a-G	8.6	11.3	0.334	1.143 (0.855-1.530)

P values were adjusted for age and gender. For abbreviations, see legends to Tables 1 and 2.

Because age has been shown to correlate with the IVD degeneration, we selected subjects from a restricted age range in an attempt to lessen the effect of this variable. Since young lumbar discs are exposed to environmental strain for less time than older discs, it is possible that genetic factors may have a specific role in the IVD degeneration in the younger population. In addition, IVD pathology (degeneration, bulging, protrusion, and extrusion) is a spectrum of disease and expression of VEGF could differ according to the stages of IVD pathology. The increase in VEGF expression has been shown to have more severe low back pain in the IVD degeneration compared with herniated disc (Lee et al., 2009). We can recognize asymptomatic individuals with IVD degeneration. Thus, *VEGF* polymorphisms could be variable according to age, stages of IVD pathology, and presence of low back pain. To minimize these possible confounders, we chose young subjects and excluded the patients with obvious disc protrusion or extrusion. On the basis of our results, we speculated that combined genotypes of *VEGF* -2578CA+AA may be associated with insufficient blood supply to the IVD via narrowing of capillaries at the disc margins of the discs or change in endplate quality due to calcification.

There are several limitations in this study. 1) This was a hospital-based case-control study, which had a relatively small sample size and had no replication study. 2) The population studied comprised only Korean individuals, and because of interethnic variability in SNP and haplotype frequency, our findings will need to be validated in other ethnic groups. 3) Comparison between the IVD degeneration group and herniated disc group are required. 4) Imaging of lumbar arteries

is necessary to compare the degree of atherosclerosis between case and control groups. 5) Our results may underestimate the true impact of individual risk factors or other genetic factors. Therefore, large-scale and replication studies are necessary to confirm and expand upon our findings.

CONCLUSION

The proposed individual *VEGF* and *eNOS* polymorphisms were not associated with the risk of IVD degeneration. However, the frequency of the combined *VEGF* genotypes of -2578CA/-634CC and -2578AA/-634CC were significantly increased in patients with IVD degeneration, suggesting that *VEGF* -2578CA/-634CC and -2578AA/-634CC could be genetic markers for IVD degeneration. In addition, the frequencies of the A-A-C-C (*VEGF* -2578/-1154/-634/936), A-A-C (*VEGF* -2578/-1154/-634), A-C-C (*VEGF* -2578/-634/-936), and A-C-T (*VEGF* -2578/-634/-936) haplotypes were significantly higher in patients than in controls, indicating that the *VEGF* -634C allele conferred risk for IVD degeneration. Our study is the first to investigate the relationship between *VEGF* and *eNOS* gene polymorphisms and IVD degeneration, and it provides important preliminary data to guide future investigations of these polymorphisms in a very common condition.

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