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ORIGINAL ARTICLE

The *IL-1β* (+3953 T/C) gene polymorphism associates to symptomatic lumbar disc herniation

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Abstract
Purpose To determine whether polymorphisms (SNPs) in the genes encoding cytokines and nitric oxide synthase (NOS) might play some role in lumbar disc herniation (LDH).
Patients and methods Case–control study in which 179 patients were retrospectively reviewed. The case group was made of 50 patients with symptomatic LDH diagnosed by MRI while the control group was made of 129 individuals undergoing routine hip or knee arthroplasty with a lifetime lack of low back pain. SNPs in the cytokine genes of IL-1 [IL-1α (−889 C/T), IL-1β (+3953 T/C)], TNF-α (−308 G/A and −238 G/A) and NOS genes [eNOS (r 27 bp, intron 4 and −786 T/C) and iNOS (22 G/A)].
Results The CC genotype and C allele of the IL-1β (+3953 T/C) SNP were significantly more frequent among LDH patients compared to controls. On the other hand, eNOS (−768 T/C) and iNOS (22 G/A) SNPs were significantly more common in the control group.
Conclusions Carriers of the CC genotype of the IL-1β (+3953 T/C) SNP were more frequent among LDH patients suggesting some potential role of the IL-1β SNP on LDH pathogenesis. The eNOS (−786 T/C) and iNOS (22 G/A) SNPs were more frequent among the control subjects, suggesting their possible protective role against LDH. Genotyping these SNPs could be useful to identify persons with an increased lifetime risk of disc herniation in whom measures to avoid LDH could be implemented.

Keywords Lumbar disc herniation · Polymorphism · Cytokine · Nitric oxide synthase

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Introduction

The prevalence of lumbar disc degeneration is variable in the general population and depends on the individuals studied and their racial background. Schmorl nodes of lumbar spine have been found in 16.4% of asymptomatic Southern Chinese in the fourth decade of life by MRI [1]. Lumbar disc bulge was observed in 84.8% and disc herniation in 18.2% of asymptomatic Americans older than 55 while less than 10% of Spanish patients with low back pain showed lumbar end plate erosions and spondylolisthesis by MRI [2, 3]. Older reports estimated the incidence of lumbar disc herniation (LDH) detected by CT in Spain at around 2% [4]. LDH occurs when the soft central *nucleus pulposus* of a lumbar intervertebral disc is displaced from its usual anatomical position, possibly causing nerve root compression and symptoms of low back pain and sciatica. Most frequently affected are the intervertebral spaces L4–L5 and L5–S1. LDH is a common motive for sick leave and a significant medical, social and economic burden.

Epidemiological studies in twins have shown that the most determining individual factor for suffering intervertebral disc degeneration is a family history of this condition [5, 6]. Environmental factors such as mechanical variables, lifestyle factors such as body weight or even smoking play an important role in LDH as well [7, 8]. The patient's genetic makeup could, therefore, be the factor that under similar environmental conditions determines that some individuals develop this condition while others do not. Among these genetic factors that lead to variation in the response of an organism to a similar environmental stimulus, we find gene polymorphisms (GP).

A GP is an allelic variation of a gene that exists in stable form in a population at a frequency of at least 1%. Two types of GP have been described: variable number of tandem repeat polymorphisms (VNTR) and single nucleotide polymorphisms (SNP), which appear more frequently. Its phenotypic expression has no specific clinical effect but a GP determines, e.g., that a person is responsive or not to a given drug or susceptible to suffer a given disease. Polymorphisms differ from mutations in that the latter are much less common and are generally associated with diseases.

It is well established that cytokines play a role in intervertebral disc degeneration and, specifically, interleukin (IL)-1 has been incriminated in this process among other factors that mediate inflammation. Hence, a predisposition to disc degeneration could be related to the expression of GP in the genes that code for such mediators, particularly IL-1 [9, 10].

Nitric oxide (NO) is a small molecule of short half-life that is generated by the enzyme nitric oxide synthase (NOS). The role of NO as a physiological mediator has been the topic of intense investigation. It is known that NO regulates arterial pressure, platelet aggregation, wound repair and apoptosis, and is involved in the response to an allograft, ulcerative colitis and gastrointestinal disorders. It also participates in the processes of interest in Orthopaedic Surgery such as inflammation, arthritis, osteoporosis, sepsis, ligament repair and aseptic loosening of joint prostheses [11, 12]. It was reported that NO mediates the change of proteoglycan synthesis in the human lumbar intervertebral disc in response to hydrostatic pressure [13]. As for cytokines, the expression of NO may vary according to the GP carried by an individual.

The aim of this work was to determine a potential association between LDH and different SNPs of cytokines [*IL-1α* (−889 C/T); *IL-1β* (+3953 T/C); tumor necrosis factor (*TNF*) [(−308 G/A and −238 G/A)], endothelial nitric oxide synthase (*eNOS*) [(27 bp repeat in intron 4) and (−786 T/C)]; and inducible nitric oxide synthase (iNOS) (*iNOS* 22 G/A). To prove this hypothesis, a retrospective case–control study was designed in which 50 patients with MRI-confirmed symptomatic LDH and 129 individuals undergoing routine hip or knee arthroplasty at the Hospital Universitario Central de Asturias with a lifetime lack of low back pain were genotyped for the above SNPs.

Patients and methods

Patients

One hundred and seventy nine patients were recruited from the Orthopaedic Surgery and Traumatology Department of the Hospital Universitario Central de Asturias (HUCA) between July 2007 and December 2008.

The case group was made of 50 patients, 21 women (43%) and 29 men (58%) with several clinical symptoms suggestive of LDH and the condition confirmed by Magnetic Resonance Imaging (MRI). LDH symptoms were those described by the Spine Center of the University of California San Francisco Medical Center as follows: (1) lower back pain, (2) pain, weakness or tingling in the legs, buttocks and feet, (3) difficulty in moving the lower back, (4) problems with bowel, bladder or erectile function in severe cases [14]. LDH MRI findings were extension of the disk beyond margins of adjacent vertebral bodies. The mean age of the case group was 43.9 ± 11.9 years [range 23–77]. Fifteen of them (30%) underwent surgery and the LDH diagnosis was confirmed at the operation room while the 35 remaining LDH patients were on non-surgical therapy.

The control group was made of 129 patients who had been admitted to the Orthopaedic Surgery and Traumatology Department for primary hip or knee arthroplasty. This control group was made of 44 men (34.1%) and 85 women (65.9%) who has a lifetime lack of symptoms suggesting LDH. Their mean age was 68.7 ± 9.2 years [range 25–85].

Both cases and controls were from a homogeneous Caucasian population in Hardy–Weinberg equilibrium and lived in the same region (Asturias). Each participant was required to sign an informed consent form, which had been approved by the Medical Ethics Committee of the HUCA.

Genotypes

Blood samples (10 mL) were withdrawn from each subject into a vacuum tube containing K3-ethylenediaminetetraacetic acid (EDTA) as anticoagulant by puncturing a peripheral vein in the arm.

The next part of the study was performed at the Biochemistry and Molecular Biology laboratory, Oviedo University Medical School, where the necessary DNA was extracted and isolated to genotype the following SNPs: *IL-1α* (−889 C/T); *IL-1β* (+3953 T/C); *TNF-α* [(−308 G/A and −238 G/A); *eNOS* [(27 bp repeat in intron 4) and (−786 T/C)]; and *iNOS* (iNOS 22 G/A).

DNA was first isolated from the blood sample leucocytes by the saline precipitation method described by Miller and Polesky [15]. Next, the DNA fragment containing the polymorphic sequence was amplified by PCR. The products of amplification were visualized by 2% w/v agarose gel electrophoresis. Based on prior knowledge of the size of the possible resultant alleles and their electrophoretic patterns, the corresponding genotype for each SNP was determined. The primers used in each case are provided in Table 1 along with the annealing temperatures and restriction enzymes used.

Statistical analysis

Data analysis was performed using the programs SPSS Software, version 15.0 (Chicago, IL, USA) and BMD Statistical Software Ltd. (Cork, Ireland). Genotype frequencies in the two groups were compared using the Chi-squared test. Odds ratios (OR) and their 95% confidence intervals (CI) were also calculated. The level of significance was set at $p < 0.05$. Yates' correction and Mantel–Haenszel tests were used when necessary.

Results

The genotypic and allelic frequencies observed for the different SNPs in LDH patients and controls are shown in Tables 2, 3, 4 and 5.

Interleukin-1β SNP: IL-1β (+3953 T/C)

Carriers of the homozygous polymorphic CC genotype were threefold more frequent among the LDH cases than in controls ($\chi^2 = 3.1$; OR = 3.65 [0.66–21.54]; $p = 0.008$) (Table 2). In addition, the C allele was detected more frequently in LDH patients than controls ($\chi^2 = 4.12$; OR = 1.7 [0.98–2.93]; $p = 0.042$) (Table 3).

Endothelial nitric oxide synthase SNP: eNOS (−786 T/C)

The polymorphic CC genotype was fivefold more frequent among controls than in the LDH patients ($\chi^2 = 13.88$; OR (95% CI) = 0.12 [0.03–0.43]; $p = 0.0002$) (Table 4). The C allele of the SNP NOS3 (−786 T/C) was also significantly more frequent among the controls compared to the LDH patients ($\chi^2 = 4.1$; OR (95% CI) = 0.6 [0.35–1.01]; $p = 0.042$) (Table 5).

Inducible nitric oxide synthase SNP: iNOS (22 G/A)

The polymorphic AA genotype was observed in 14.7% of the control subjects and in none of the patients with a

Table 1 Genetic polymorphisms examined in the present study and primers used with their corresponding annealing temperatures, restriction enzymes and amplification product sizes

Polymorphism	Primers (sense/antisense)	Annealing temperature (°C)	Restriction enzyme	PCR product size	Fragment sizes
IL-1α (−889 C/T)	5′-ATCACACCTAGTTCATTTCCTCTATTTA-3′	58	*Nco*I	195 bp	195 bp (T)
	5′-GATTTTTACATATGAGCCTTCCATG-3′				166 + 29 bp (C)
IL-1β (+3953 T/C)	5′-CTCAGGTGTCCTCCAAGAAATCAAA-3′	60	*Taq*I	194 bp	194 bp (C)
	5′-GCTTTTTTGCTGTGAGTCCCG-3′				108 + 86 bp (T)
TNF-α (−308 G/A)	5′-GCAATAGGTTTTGAGGGCCAT-3′	58	*Nco*I	147 bp	122 + 25 bp (G)
	5′-GGGACACACAAGCATCAAG-3′				147 bp (A)
TNF-α (−238 G/A)	5′-AAACAGACCACAGACCTGGTC-3′	58	*Bam*HI	154 bp	110 + 44 (G)
	5′-CTCACACTCCCCATCCTCCCGGATC-3′				154 bp (A)
eNOS (27 bp, intron 4)	5′-CTATGGATGTGCCTTGGCTGGAGG-3′	63	–	22 bp (6rep)	–
	5′-TCGCCTCAAGGGACCCGCCA-3′			195 bp (5rep)	
				168 (4rep)	
eNOS (−786 T/C)	5′-TGGAGAGTGCTGGTGACCCCA-3′	62	*Msp*I	180 bp	140 + 40 bp (T)
	5′-CTGTCCCACCCCCACCTCCG-3′				90 + 50 + 40 bp (C)
iNOS (22 G/A)	5′-GCTGAATCTGAGTTGATGAACAGATC-3′	60	*Nco*I	140 bp	120 + 20 bp (G)
	5′-CTCCCGGGATCACACGCCCA-3′				140 bp (A)

Table 2 Polymorphisms in the cytokines-encoding genes *IL-1α* (−889C/T), *IL-1β* (+3953 T/C), *TNF-α* (−308 G/A) and (−238 G/A) in patients with lumbar disc herniation (LDH) and control patients

Gene (%)	Genotype frequency	LDH ($n = 50$)	Controls ($n = 129$)	Pearson χ^2	Odds ratio (95% CI)	p
IL-1-α (−889 T/C)	TT	3 (6)	5 (3.9)	0.38	1.58 (0.29–8.02)	0.54
	CT	25 (50)	61 (47.3)			NS
	CC	22 (44)	63 (48.8)			NS
IL-1-β (+3953 T/C)	CC	4 (8)	3 (2.3)	3.10	3.65 (0.66–21.54)	0.008*
	CT	16 (32)	50 (38.8)			NS
	TT	30 (603)	76 (58.9)			NS
TNF-α (−308 G/A)	AA	1 (2)	4 (3.1)	0.16	0.64 (0.03–6.3)	0.69
	AG	16 (32)	42 (32.6)			NS
	GG	33 (66)	83 (64.3)			NS
		($n = 49$)	($n = 122$)			
TNF-α (−238 G/A)	AA	0	0	–	–	–
	AG	5 (10)	9 (7.38)	0.33	1.4 (0.39–4.90)	0.57
	GG	45 (90)	113 (92.62)			

* p value was calculated by the two-tailed Fisher's exact test

Table 3 Allele frequencies of polymorphisms of the cytokines-encoding genes *IL-1α* (−889 C/T), *IL-1β* (+3953 T/C), TNF-α (−308 G/A and 238 G/A) in patients with lumbar disc herniation (LDH) and controls

Gene (%)	Allele frequency	LDH ($n = 50$)	Controls ($n = 129$)	Pearson χ^2	Odds ratio (95% CI)	p
IL-1-α (−889 T/C)	C	69/100 (0.69)	187/258 (0.73)	0.43	1.2 (0.69–2.02)	0.51
	T	31/100 (0.31)	71/258 (0.27)			
IL-1-β (+3953 T/C)	C	32/100 (0.32)	56/258 (0.22)	4.12	1.7 (0.98–2.93)	0.042*
	T	68/100 (0.68)	202/258 (0.78)			
TNF-α (−308 G/A)	A	18/100 (0.18)	50/258 (0.19)	0.09	0.91 (0.48–1.72)	0.77
	G	82/100 (0.82)	208/258 (0.81)			
		($n = 50$)	($n = 122$)			
TNF-α (−238G/A)	A	5/100 (0.05)	9/244 (0.03)	0.31	1.37 (0.39–4.63)	0.58
	G	95/100 (0.95)	235/244 (0.97)			

* p calculated by the Mantel–Haenszel test

Table 4 Polymorphisms in *eNOS* (27 bp repeat, intron 4), and (−786 T/C) and *iNOS* (22 G/A)-encoding genes in patients with lumbar disc herniation (LDH) and controls

Gene (%)	Genotype	LDH ($n = 50$)	Controls ($n = 129$)	Pearson χ^2	Odds ratio (95% CI)	p
NOS3 (27 bp, intron 4)	44	0 (0)	3 (2.3)	1.18	ND	0.28
	45	10 (20)	28 (21.7)			NS
	55	40 (80)	97 (75.2)			NS
	65	0 (0)	1 (0.8)			
NOS3 (−786 T/C)	CC	3 (6)	45 (34.9)	13.88	0.12 (0.03–0.43)	0.0002*
	CT	30 (60)	55 (42.69			NS
	TT	17 (34)	29 (22.5)			NS
NOS2 (exon 22)	AA	0 (0)	19 (14.7)	6.76	ND	0.0009*
	GA	35 (70)	63 (48.8)			
	GG	15 (30)	47 (36.4)			

Confidence intervals could not be calculated in this case and are only provided for the relative risk as: RR (95% CI) = 0.87 (0.16–4.81)

* p value was calculated by the Yates correction test

Table 5 Allele frequencies of polymorphisms in eNOS (27 bp repeat, intron 4), and (786 T/C) and iNOS (22 G/A)-encoding genes in patients with lumbar disc herniation (LDH) and controls

Gene (%)	Allele frequency	LDH ($n = 50$)	Controls ($n = 129$)	Pearson χ^2	Odds ratio (95% CI)	p
NOS3 (27 bp, intron 4)	4	10/100 (0.1)	34/258 (0.14)	0.68	0.73 (0.32–1.62)	0.41
	5	90/100 (0.9)	222/258 (0.86)			
	6	0/100 (0)	2/258 (0.0)			
NOS (−786 T/C)	C	36/87 (0.41)	145/258 (0.66)	4.1	0.6 (0.35–1.01)	0.042*
	T	47/87 (0.59)	113/258 (0.44)			
NOS2 (exon 22)	A	35/100 (0.35)	101/258 (0.39)	0.53	0.84 (0.5 –1.39)	0.47
	G	65/100 (0.65)	157/258 (0.61)			

* p calculated by the Mantel–Haenszel test

herniated disc, the comparison being significant ($\chi^2 = 6.76$; $p = 0.0009$) (Table 4). No significant differences were detected when allele frequencies of this SNP between the two groups were compared ($p = 0.47$; Table 5).

Other SNPs

No significant differences were observed when the genotypic and allelic frequencies of the remaining SNPs genotyped in LDH cases and controls were compared.

Discussion

The present study shows an association between the carriage of the *IL-1β* (+3953 T/C) SNP and symptomatic LDH Spanish patients. Several reports have found an association between different SNPs and intervertebral disc disease so far [6, 9, 17–27]. Due to their proinflammatory role, variations in genes codifying cytokines were studied first. Of them, only the *IL-1α* (−889 C/T), and *IL-1β* (+3953 T/C)] were clearly associated with intervertebral disc disease [9, 17, 18, 21]. Our results agree with those of Solovieva et al. observed in a Finnish cohort in which the carriage of the *IL-1β* (+3953) T allele correlated not only with intervertebral disc degeneration, but also with the number of days of low back pain [9, 18]. However, we could not find an association between the *IL-1α* (−889 C/T) SNP and LDH in our Spanish cohort that others have reported not only in Finnish, but also in Chinese patients [9, 19, 21]. There is a linkage disequilibrium between *IL-1α* and *IL-1β* genes, both located in the long arm of chromosome 2, so it is difficult to be sure whether these reported associations are specific for one or the other gene.

The link between cytokines and LDH is still poorly understood. *IL-1β* SNP associates with increased "in vitro" expression of IL-1β [22]. IL-1 induces the expression of other proinflamatory enzymes such as NOS, matrix metalloproteases (MMPs) and cyclooxigenases (COX) that might destroy proteoglycans causing disc degeneration. This hypothesis is supported by the fact that Takahashi et al. [16] detected the presence of IL-1α, IL-1β, IL-6, TNF-α and prostaglandin E2 in the disc tissue of almost one hundred patients with a herniated disc. *MMPs* and *COX* SNPs have been associated with lumbar disc degeneration as well [23, 24].

The *eNOS* (−786 T/C) and *iNOS* (22 G/A) SNPs were more frequent among the control subjects with a lifetime lack of LDH symptoms, suggesting a potential protective role against LDH development. Ours is the first report of an association between a *NOS* SNP and protection against LDH in the world literature.

The *IL-1β* SNP carried by only 8% of LDH patients in our cohort cannot explain but a minor part of the intervertebral disc disease pathogenetic mechanisms. The genetic component associated with intervertebral disc disease is of a highly complex nature. A large number of biological mediators are involved in this pathologic process not only interleukins, but also NOS, MMPs and COX. Genes encoding one of the three alpha chains of type IX collagen, the major collagen component of hyaline cartilage or the vitamin D receptor seems to play a role as well. The multiple SNPs existing for each of the genes that encode these molecules may contribute to the disease in varying measure of protect against its development. Thus, the final outcome is determined by cumulative expression of the cluster of polymorphic genes, leading to variation in the effects observed in the subset of individuals with disc herniation. Environmental and occupational factors play an additive or role in association to the genetic background in vertebral disc degeneration, such as the effect of weight lifting or whole-body vibration on the carriage of the *IL-1α* (−889 T/C) allele or the synergistic effect of obesity on the carriage of the COLA9A3 SNP [17, 19, 27].

Caution is needed in the interpretation of our data. Ours is a case–control, relatively small, only Caucasian, retrospective and non-aleatorized cohort. Larger populations of

other non-Caucasian ethnic backgrounds must be genotyped for this *IL-1β* SNP. In addition case and control groups were not matched for age or gender with a predominance of older female in the control group, made of individuals undergoing orthopaedic prosthesis surgery. Control individuals did not have a spine MRI done ruling out LDH. However, they reported a lifetime lack of low back pain. The above differences could bias our findings. However, the mean old age of the control individuals (68.7 years), with their active laboral life finished, made unlikely the further development of LDH.

Conclusions

The clinical meaning of the association between the *IL-1β* SNP and LDH, confirming similar findings in other international cohorts, requires further investigation including the determination of serum levels of IL-1β in carriers of this *IL-1β* SNP, in both LDH cases and controls and its correlation with other already reported SNPs with intervertebral disc involvement in patients with active or sedentary life-styles. If the association observed here between the *IL-1β* SNP and LDH is confirmed, then all subjects whose work or other activity might increase their risk of suffering disc pathology should be genetically screened for this SNP. Preventive measures could be taken in carriers of this genotype such as redesigning their sports or work life. If used as a tool for early diagnosis and prevention, the analysis of SNPs associated with skeletal pathologies could be a promising approach.

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Conflicts of interest The authors declare no conflicts of interest.

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