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The Role of Collagen IX Tryptophan Polymorphisms in Symptomatic Intervertebral Disc Disease in Southern European Patients

Stefanos N. Kales, MD, MPH,*†‡ Athena Linos, MD, MPH,§ Christos Chatzis, MD,§ Yang Sai, PhD,* Maria Halla, BSc, MSc,§ Georgios Nasioulas, PhD,‖ and David C. Christiani, MD, MPH, MS*†¶

Study Design. We conducted a cross-sectional, genotyping study of intervertebral disc disease patients and controls.

Objectives. To determine the contribution of COL9A2 and COL9A3 Tryptophan polymorphisms to intervertebral disc disease development in a genetically heterogeneous, Southern European population compared to previous Finnish studies.

Summary of Background Data. The COL9A2 and COL9A3 genes encode the α2 and α3 chains of Collagen IX. Recent Finnish studies suggest that a tryptophan polymorphism in the COL9A2 gene (Trp2) results in hereditary intervertebral disc disease, whereas a similar tryptophan mutation in COL9A3 (Trp3) conveys a 3-fold risk of intervertebral disc disease.

Methods. We studied 105 symptomatic patients with radiographically and/or surgically proven lumbar (98%, n = 103) or cervical (2%, n = 2) intervertebral disc disease and 102 age-matched controls without spinal complaints from hospitals in Athens, Greece. Intervertebral disc disease was defined as significant disc herniation resulting in persistent back or leg pain. We genotyped all patients for COL9A2 and COL9A3 allele variations using a polymerase chain reaction-based technique.

Results. None of our patients had the Trp2 allele. Consistent with previous Finnish findings, more Greek intervertebral disc disease cases (8.6%) than controls (4.9%) had at least 1 Trp3 allele, but this difference did not reach statistical significance ($P = 0.293$). The allele frequency of the Trp3 mutation was significantly higher among previously studied Finnish patients with intervertebral disc disease (12.3%) than among the Southern European patients with intervertebral disc disease in our study (4.3%), $P = 0.001$.

Conclusions. The differences in Trp allele frequency we found between Greek and Finnish patients with intervertebral disc disease most likely represent true differences in polymorphism prevalence between the respective populations. The 2 previously described Trp alleles in COL9A2 and COL9A3 are likely to be less significant susceptibility factors for intervertebral disc disease development in Southern European populations. [Key words: intervertebral disc disease, back pain, collagen, genetics, familial predisposition] **Spine 2004;29:1266–1270**

Although various environmental, ergonomic and anthropometric risk factors are associated with symptomatic intervertebral disc disease (IVDD), increasing evidence in the literature suggests that heredity also plays a role.[1–12] Several studies[6,7,9–12] have found familial predisposition to IVDD, including early-onset sciatica and lumbar disc herniation, whereas others have found that genetic mutations in collagen IX and aggrecan can cause age-related disc degeneration and herniation in mice.[4,13]

Recently, Annunen *et al*[2] identified a putative disease-causing sequence variation in the α2 chain of collagen IX (COL9A2) that converted a codon for glutamine or arginine to 1 for tryptophan in 6 of 157 cases of sciatica, but none of 174 controls. Family members in 4 of these 6 cases were also studied. All 20 determined to carry this tryptophan (Trp2) allele were examined clinically and with imaging studies and were found to have IVDD. Subsequently, the same group showed that another tryptophan allele (Trp3) in the α3 chain of collagen IX, COL9A3, conveyed a 3-fold risk of IVDD.[8] This second allele, Trp3, was found in 23% of Finnish patients with IVDD, but only 9.3% of controls. These impressive results were found in Finnish patients who are relatively genetically homogeneous.[5] Because there has been little study of these polymorphisms elsewhere, we decided to investigate the contribution of collagen IX mutations to IVDD in a representative Southern European population that was genetically heterogeneous.

Materials and Methods

Patients. Intervertebral disc disease cases were symptomatic patients with surgically or radiologically proven herniated

From the *Occupational Health Program, Harvard School of Public Health, Boston, Massachusetts, †Harvard Medical School, Boston, Massachusetts, ‡Cambridge Health Alliance, Cambridge, Massachusetts, §University of Athens Medical School, Athens, Greece, ‖Hygeia Hospital, Department of Molecular Biology, Athens, Greece, and ¶Pulmonary and Critical Care Program, MA General Hospital, Boston, Massachusetts.

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Address correspondence to Stefanos N. Kales, MD, MPH, Occupational Medicine, Cambridge Health Alliance, 1493 Cambridge Street, Cambridge, MA 02139, USA; E-mail: skales@challiance.org; address reprint requests to David C. Christiani, MD, MPH, MS, Professor and Director, Occupational Health Program, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA; E-mail: dchris@hohp.harvard.edu

discs of the lumbar (98%, n = 103) or cervical (2%, n = 2) spine from the orthopedic and neurosurgical departments of several major teaching hospitals in Athens, Greece. The vast majority (83%) of the cases and controls were recruited from a single institution, Hygeia Hospital. During the study period, all such patients less than 60 years old were invited to participate. Minimal inclusion criteria for the cases were the presence of disc herniation, with or without nerve root impingement, or compression of the spinal cord, producing persistent back or leg pain unrelieved by anti-inflammatory drugs. The criteria for surgical intervention included the following: disabling pain for greater than 2 months accompanied by compatible imaging findings as above; foot drop; cauda equina syndrome; and cord compression syndrome due to disc disease.

Due to the high prevalence of aging-associated degenerative disc changes that are less likely to be genetically determined, we excluded older patients. Conversely, we hypothesized that persons with earlier-onset sciatica are more likely to have a genetic predisposition.

Control participants were recruited from patients with non–spine-related/nonarthritic problems and visitors to nonsurgical, nonorthopedic units from the same hospitals. Exclusion criteria were age greater than 59 years old, familial relation to any of the cases, and a history of seeking medical attention for back pain. Controls were serially matched to the cases by age (±5 years). Previous epidemiologic studies in Greece have demonstrated that the use of patients with unrelated conditions and visitors from the same hospitals results in similar demographic, socioeconomic, and ethnic backgrounds among the cases and controls.[14,15]

Written informed consent was obtained from all participants (cases and controls) before participation in the study. The Ethics Committee of the Hygeia Hospital, Athens, Greece, and the Human Subjects Committee of the Harvard School of Public Health, Boston, MA, approved the study protocol. Of the 210 original participants, we excluded 3 patients who were not of Greek origin, leaving 105 IVDD cases and 102 controls of Greek descent.

Questionnaire. All participants were administered a prestructured questionnaire by a trained interviewer. The questionnaire included detailed information on the participant's personal, family, and back pain history as well as diagnostic data and functional status.

Genotyping. Twenty-five milliliters of blood were drawn from each patient into tubes of 10 mL, 10 mL, and 5 mL and transferred to the molecular biology laboratory of the Hygeia Hospital. Genomic DNA was extracted from clotted blood using the PUREGENE® DNA isolation kit (Genra Systems, Minneapolis, MN) from 3 mL whole blood per our previously described method.[16] The isolated DNA samples were stored at 2 to 4 C until transfer to the molecular epidemiology laboratory of the Harvard School of Public Health, where further procedures analyzed the DNA for COL9A2 and COL9A3 genotype variations.

The DNA amplification used 100 to 300 ng genomic DNA in a final reaction volume of 25 μL. For each assay, amplification reactions were performed with the QIAGEN PCR System (QIAGEN, Germany) containing 1× Hot Start Buffer, 2.5 mmol/L $MgCl_2$, 1× QIAGEN Q-solution, 200 μmol/L dNTPs, 0.2 μmol/L forward and reverse primers, and 0.5 U of HotStar *Taq* enzyme. For the *COL9A2* polymorphism, amplification conditions were as follows: 95 C for 15 minutes, 30 cycles of 95 C for 1 minute, 61 C for 1 minute, 72 C for 2 minutes, and a final extension at 72 C for 8 minutes. For the *COL9A3* polymorphism, the amplification conditions were 30 cycles of 95 C for 45 seconds, 63 C for 45 seconds, and 72 C for 1 minute.

Sequence variations in COL9A2 were determined from genomic DNA using a polymerase chain reaction (PCR)-pyrosequencing technique with negative control samples and restriction fragment length polymorphism (RFLP) analyses. Primers were designed to determine the sequence variations in COL9A2 described in the gene-wide screen analysis performed by Annunen *et al*,[2] specifically, the presence of the Trp substitution in position 326 of COL9A2 (exon 19, TGG for Trp), as well as the expected wild-type sequences (CAG for Glu, and CGG for Arg). Subsequently, standard RFLP analyses were performed.

Similar subsequent procedures were performed for COL9A3 using a PCR-pyrosequencing method with negative control samples. Primers were designed to determine the sequence variations in COL9A3 described by Paassilta *et al*,[8] specifically, the presence of the Trp substitution in position 103 of COL9A3 (exon 5, TGG for Trp), as well as the expected wild-type sequences (CGG for Arg and CAG for Glu).

Pyrosequencing reactions were performed according to the manufacturer's instructions (Pyrosequencing, Uppsala, Sweden) with minor modifications as previously described.[17] Briefly, 5 μL (10 μg/μL) Streptavidin-coated Dynabeads (Dynal, Oslo, Norway) was added to 25 μL 2× binding-washing buffer (10 mmol/L Trizma base, 2 M NaCl, 1 mmol/L EDTA, 0.1% Tween 20, pH 7.6), and then mixed with the 25 μL PCR product, and the plates were sealed and shaken at 65 C for 20 minutes. Solidphase (bound to beads) samples were transferred consecutively to pyrosequencing plates containing 0.5 M NaOH (for 1 minute), 1× annealing buffer (200 mmol/L Trizma Base, 50 mmol/L magnesium acetate, pH 7.6), and 1× annealing buffer containing 15 pmol sequencing primer. Following the last step, samples were heated at 80 C for 2 minutes and then cooled for approximately 15 minutes before pyrosequencing analysis. Pyrosequencing was then performed at room temperature on an automated pyrosequencing 96 instrument (Pyrosequencing, Uppsala, Sweden). For each assay, a template-only control, a sequencing primer-only control, a biotinylated primer-only control, and a combination of sequencing and biotinylated primers were evaluated to verify that each had negligible effect on baseline signal.

Statistical Analysis. Statistical analyses were performed using SPSS.[18] Independent *t* tests were used to compare the differences in mean values. The "Crosstabs" procedure was used to calculate proportions and generate χ^2 statistics between IVDD cases and controls. The Fisher exact test was also used as appropriate when any expected cell count was less than 5. The level of significance was 0.05 and two-tailed for all tests.

■ Results

The background characteristics of the IVDD cases and the controls without spinal complaints are described in Table 1. The vast majority of the cases (95%) had symptoms of sciatica and significant functional limitations. Lower extremity symptoms were present in 98% of the surgical cases and more frequently reported as compared

Table 1. Characteristics of the Study Groups: IVDD Cases and Controls

	IVDD Cases (n = 105)	Controls (n = 102)	*P* Value
Age (mean ± SD)	39.0 ± 8.5	35.1 ± 7.9	0.001
BMI (mean ± SD)	26.0 ± 4.2	25.5 ± 4.9	0.452
Sex, % male (n)	65% (68)	46% (47)	0.007
History of significant back pain* [% (n)]	100% (105)	0% (102)	<0.001
Severe pain in the last month	66% (68/103)		
Any lower extremity symptom	95% (100)		
Lower extremity pain	79% (83)		
Lower extremity paresthesia	62% (65)		
Lower extremity weakness	20% (21)		
Absence from work	84% (82/97)		
Limitation carrying shopping bags	81% (84/104)		
Limitation walking several blocks	75% (78/104)		
Limitation bathing/dressing	50% (52/104)		
Radiologic confirmation of IVDD [% (n)]	95% (100)	—	—
CT	36% (38)		
MRI	36% (38)		
CT and MRI	23% (24)		
Surgical intervention [% (n)]	62% (65)	—	—
Type of surgical intervention			
Microdiscectomy	37% (39)		
Discectomy	22% (23)		
Laminectomy	2% (2)		
Discectomy and vertebral fusion	1% (1)		

* Back pain requiring medical attention.
IVDD = intervertebral disc disease; BMI = body mass index; CT = computed tomography; MRI = magnetic resonance imaging; — = data not available.

Table 2. Possible Genetic Factors for IVDD Among the IVDD Cases and Controls

	IVDD Cases (n = 105)	Controls (n = 102)	*P* Value
Family history of IVDD*	47% (49)	32% (33)	0.035
One Trp2 allele†	0% (0)	0% (0)	—
One Trp3 allele‡	8.6% (9)	4.9% (5)	0.293
One Trp3 allele‡ (cases restricted to those with lower extremity symptoms)	9.0% (9/100)	4.9% (5)	0.252
One Trp3 allele‡ (Cases restricted to those "limited a lot in activities of moderate intensity")	12.1% (7/58)	4.9% (5)	0.122§
One Trp3 allele‡ (males only)	8.8% (6/68)	2.1% (1/47)	0.237§

* At least 1 parent and/or sibling with a history of IVDD.
† Trp substitution in position 326 of the α2 chain of collagen IX.
‡ Trp substitution in position 103 of the α3 chain of collagen IX.
§ Fisher exact test.
IVDD = intervertebral disc disease; — = data not available.

to the nonsurgical cases including: pain ($P = 0.057$), numbness ($P = 0.003$), sensory deficit ($P = 0.048$), and weakness ($P = 0.055$). Surgical cases also reported significantly more functional limitations in the following: activities of moderate intensity ($P = 0.001$), carrying shopping bags ($P < 0.001$), and walking several blocks ($P < 0.001$).

The frequencies of possible genetic factors for IVDD for both groups are shown in Table 2. Although a family history of IVDD was more frequent among the patients with IVDD than controls, none of the patients had the Trp2 mutation in COL9A2. Because of this distribution, no further case-control analysis was pursued for Trp2.

Although almost twice as many IVDD cases as controls had the Trp3 mutation in COL9A3, and the difference was 4-fold among male patients, these differences did not reach statistical significance. The prevalence of Trp3 was highest when the cases were restricted to those most functionally limited during activities of moderate intensity. Further stratified analyses both between IVDD cases and controls and within IVDD cases were performed for factors possibly associated with genetically determined IVDD. These included a family history of IVDD, age less than 45 years old, nonobese patients (body mass index less than 30), gradual onset of pain (as opposed to sudden onset), history of surgical intervention, and an occupation not requiring lifting. None of these analyses yielded significant results, but were limited by the small number of patients with the Trp3 allele.

Table 3 compares the Trp2 and Trp3 allele frequencies for Greek and Finnish patients with IVDD. The allele

Table 3. Comparison of the Allele Frequencies at COL9A2, Position 326 and COL9A3, Position 103 in Greek and Finnish Patients With IVDD

	Greek Patients With IVDD [% (n) alleles]	Finnish Patients With IVDD[2,8] [% (n) alleles]	*P* Value Overall (*P* Value Trp Allele, Yes or No)*
COL9A2, position 326			
Trp	0 (0)	1.9 (6)	0.075 (0.088)*
Gln	78 (164)	72.6 (228)	
Arg	22 (46)	25.5 (80)	
Total	210	314	
COL9A3, position 103			
Trp	4.3 (9)	12.3 (42)	0.003 (0.001)*
Gln	1.0 (2)	0.3 (1)	
Arg	93.8 (197)	87.4 (299)	
Indeterminate	1.0 (2)	—	
Total	210	342	

* Fisher exact test.
IVDD = intervertebral disc disease; — = data not available.

frequency of Trp3 was significantly higher among the previously studied Finnish patients with IVDD (12.3%) than among the Greek patients with IVDD in our study (4.3%), $P = 0.001$.

■ Discussion

No Greek patients had the Trp2 allele of COL9A2, and although almost twice as many IVDD cases as controls had the Trp3 mutation in COL9A3, the difference was not statistically significant. Further analyses demonstrated that Trp2 and Trp3 allele frequencies were less frequent among Greek patients with IVDD compared to previously studied Finnish patients with IVDD and similar to the frequencies observed in Finnish controls. Therefore, our study suggests that the relative contribution of Trp2 and Trp3 mutations to the development of IVDD is small, if any, in the Greek population and significantly less than in Finland.

The differences in Trp allele frequency we found between Greek and Finnish patients with IVDD most likely represent true differences in polymorphism prevalence between the respective populations. The differences are unlikely to be due to the misclassification of patients with non–disc-related spinal complaints as suffering from IVDD, and they are also not likely to be due to differences in ascertainment of IVDD. In the present study and the Finnish investigations, cases and controls were defined in similar fashions. The Finnish patients had at least 1 month of sciatica, as well as physical examination and magnetic resonance imaging (MRI) findings.[2,8] Similarly, our patients with IVDD were all symptomatic, and 95% reported symptoms consistent with sciatica with a high prevalence of functional limitations in activities of daily living. They were recruited from orthopedic and neurosurgical services, had radiologic and/or surgical confirmation of IVDD, and 62% had undergone surgical intervention. In addition, the lower tryptophan polymorphism prevalences in the Greek patients with IVDD are unlikely to be due to dilution with cases of age-related degenerative disease because we excluded patients over 59 years old. Conversely, the Finnish studies included patients up to 78 years old.[2,8]

A more likely explanation for the higher prevalence of the Trp alleles in the Finnish population is its relative homogeneity. In fact, all patients with IVDD in the Finnish studies were drawn from the same geographic region of Finland.[8] In addition, it is possible that IVDD in Finnish patients with the Trp 3 allele is a result of an interaction with other homogeneous genetic factors common among the Finnish. Southern Europe, on the other hand, sits at the junction of Western and Eastern Europe, the Middle East and North Africa, where populations have mixed for several thousand years. The current population of Athens is largely derived from migrants from all of the country's various regions and islands and their descendants. Thus, although we excluded participants found to be of foreign origin, the remaining Greek patients would be expected to have considerable genetic heterogeneity, similar to many other populations in Southern Europe.

In one of the few other studies of Trp2, Wrocklage *et al*[19] identified Trp2 in only 3 of 250 German patients with IVDD (1.2%). This is roughly one-third of the prevalence in Finnish patients. They did not study any controls; most surprisingly, all 3 German patients with Trp2 were older than 60. Two were over 70 years old, and all lacked a prior history of disc herniation, suggesting late-onset degenerative disease. Therefore, the association of Trp2 with genetically determined IVDD is uncertain in the German series.

Although we did not find significant associations of IVDD with Trp2 or Trp3, genetic factors are still likely to play a role in the development of symptomatic disc disease in genetically heterogeneous populations as well. In agreement with Simmons *et al*,[11] who found 45% of patients in a Buffalo, New York, hospital undergoing surgery for IVDD to have a positive family history, 47% of our patients had a positive family history of IVDD. This prevalence was significantly higher than among the controls in the present study (32%, $P = 0.035$). Therefore, other emerging genetic risk factors such as aggrecan, metalloproteinase, and vitamin D receptor alleles may be important.[1]

There are several limitations to our study. Due to the relative low frequency of the 2 Trp alleles under investigation, we lacked the power to find very small differences between cases and controls. We would have required a sample size of 204 cases and an equal number of controls to have a statistical power of 0.80 to find the difference in Trp2 allele prevalence seen in the Finnish study (1.9% *vs.* 0%). On the other hand, our study did have a power of 0.78 to find a statistically significant difference in Trp3 allele frequency of the magnitude of the Finnish study (12.2% *vs.* 4.9%). Another limitation was that although our control patients had no history of significant back pain, we were not able to examine the controls with spine imaging. The Finnish studies shared this same limitation. On MRI, over one-third of asymptomatic individuals will demonstrate disc bulges at multiple vertebral levels.[20] Although this fact might explain the 4.9% of controls with the Trp3 allele, none of these limitations explain why both Trp alleles are less frequent among Greek patients with IVDD compared to Finnish patients. In addition, the difference between Greek and Finnish patients with IVDD for Trp3 allele frequencies was highly significant.

In summary, the 2 Trp alleles of COL9A2 and COL9A3 previously described as contributing to the development of IVDD in Finland are not likely to be major susceptibility factors among patients of Greek descent, and probably in other Southern European populations as well. Further study of the various environmental, ergonomic, anthropometric, and hereditary risk factors asso-

ciated with symptomatic IVDD is required, especially in genetically heterogeneous populations.

■ Key Points

- The current cross-sectional study examined Greek patients with IVDD and age-matched controls for the presence of Tryptophan polymorphisms in the COL9A2 (Trp2) and COL9A3 (Trp3) genes, which were recently associated with IVDD in Finland.
- None of our patients had the Trp2 allele. Prevalence of the TRP3 allele was not significantly higher in Greek cases than controls.
- The frequency of Trp3 was significantly lower in Greek patients with IVDD than among the previously studied, genetically homogeneous Finnish cohort.
- The Trp2 and Trp3 alleles are not likely to be major susceptibility factors for IVDD development in genetically heterogeneous Southern European populations.

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