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Analysis of *FokI* Polymorphism of Vitamin D Receptor Gene in Intervertebral Disc Degeneration

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Aim: We have hypothesized a possible relationship between disc degeneration (DD) and *VDR FokI*/T2C polymorphism. *Methods:* A case–control study was performed comprising 121 Brazilian patients with confirmed DD by nuclear magnetic resonance and a control group consisting of 131 healthy patients without a history of disc cysts of the lumbar spine. Detection of *VDR FokI*/T2C polymorphism was performed using restriction fragment length polymorphism–polymerase chain reaction. The chi-square test was used to compare allele and genotype frequencies between groups, and a p-value of < 0.05 was considered statistically significant. *Results:* The results disclosed statistical difference between allele distribution among cases and controls ($p = 0.025$, odds ratio = 1.58, confidence interval = 1.07–2.32) considering *VDR FokI*/T2C polymorphism. *Conclusion:* The results showed a positive association between *VDR FokI*/T2C polymorphism and DD in Brazilian patients tested.

Introduction

The intervertebral disc is a fibrocartilaginous structure whose main function is to act as a buffer, transmitting compressive loads between vertebral bodies (Buckwalter, 1995; Miller *et al.*, 1988). The intervertebral disc consists of three main structures: the cartilaginous endplates, the central nucleus pulposus, and the annulus fibrosus located at the periphery of the disc. The intervertebral disc loses its hygroscopic properties with aging, leading to a progressive dehydration process, characterizing disc disease. From the intervertebral disc degeneration (DD), the spine begins to show progressive instability of the affected region (Inoue, 1981).

The process of DD is associated with many clinical conditions, including low back pain, which is one of the most common health problems in society, being a major cause of work absenteeism and use of health services. It is estimated that 15–20% of adults have back pain during a single year and 50–80% experience at least one episode of back pain during their lifetime (Rubin, 2007).

The precise etiology of DD is not fully understood. Until recently, it was exclusively attributed to the accumulation of environmental effects, primarily micro or macro, trauma, lifestyle, smoking, atherosclerosis, and the changes that occur in the disc with aging (Zawilla *et al.*, 2013). However, more recent research has demonstrated that the influence of these factors is moderate in DD, reinforcing the notion of genetic involvement in the etiology of the disease (Nunes *et al.*, 2007). Epidemiological studies on families and twins suggest that inheritance is the major determinant of DD (Battie *et al.*, 1995; Matsui *et al.*, 1998; Sambrook *et al.*, 1999). Several disease-associating variations have been found in a number of different genes, suggesting that intervertebral DD is a multigenetic entity (Ala-Kokko, 2002; Kalichman and Hunter, 2008; Zawilla *et al.*, 2013).

To date, several gene loci associated with human DD have been identified (Chan *et al.*, 2006). Variations in the genes involved in inflammation, extracellular matrix components, and protein metabolism have been reported as associating with DD (Kalichman *et al.*, 2008).

Vitamin D is known as a hormone that regulates calcium homeostasis and bone mineralization (Cantorna and Mahon, 2004) and can be found in two forms: ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Vitamin D, derived from the diet or the bioactivation of 7-deidrocalciferol, is inert and must be activated to exert the biological functions (Fraser and Kodicek, 1970). The hormonal form of vitamin D (1,25-2-hydroxyvitamin D3) has essential roles in endocrine functions: (1) mineralization process of bone, (2) absorption of calcium from the intestine, (3) control of calcium and phosphorus homeostasis, and (4) regulation of parathyroid hormone (Vilarino *et al.*, 2011), and it has shown antiproliferative

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and immunosuppressive effects on several cell types, for example, lymphocyte proliferation and immunoglobulin synthesis, besides inhibiting the action of proinflammatory transcription factors and the production of different cytokines, such as IL-2, IL-12, among others (Froicu *et al.*, 2003; Lehman, 2005).

Most of the biological activities of vitamin D are mediated by a high-affinity receptor that acts as a transcription factor activated by ligand—the gene for the vitamin D receptor (*VDR*), located on chromosome 12 (12q13.11) and a member of the family of steroid receptors that mediates the effects of vitamin D in regulating the transcription of multiple genes. Genetic alterations in the *VDR* gene lead to significant gene activation defects affecting calcium metabolism, cell proliferation, immune function, and others, which can be explained by changes in protein conformation (Videman *et al.*, 1998). Changes in the sequence of the gene, such as polymorphisms, may occur in the noncoding region of the gene (introns) affecting the level of gene expression and thus protein levels and the coding regions (exons) and lead to changes in the sequence of protein (Valdivielso and Fernandez, 2006; Vilarino *et al.*, 2011).

Based on this observation, a possible relationship between polymorphism *FokI* of the vitamin D receptor gene and DD has been hypothesized.

Materials and Methods

Patients

A prospective case–control study that included 121 patients in the Outpatient Clinic of the Spinal Surgery at the Hospital Estadual Mário Covas, coordinated by the Department of Diseases of the Locomotor System of the Faculdade de Medicina do ABC, Santo André/SP, Brazil. The sample includes individuals with chronic low back pain associated with degenerative intervertebral disc disease and control subjects.

The inclusion criteria for patients were as follows: (1) patients with chronic low back pain (over 3 months), (2) aged < 45 years, and (3) magnetic resonance imaging (MRI) with DD on sagittal T2. The exclusion criteria were as follows: (1) patients who underwent previous surgical treatment, (2) patients with congenital deformities of the spine, and (3) patients who refuse to sign the consent form and donate a blood sample for analysis of genomic DNA.

Considering the control group, the patients were recruited in the Clinical Laboratory of the Faculdade de Medicina do ABC and the inclusion criteria were as follows: (1) aged 20–45 years, (2) without previous surgery, (3) no history of disc hernia treatment, (4) have not been hospitalized for back pain, (5) not taking medication for back pain for more than 7 days, and (6) do not have family members younger than 45 years with disc herniation or clinical treatment for low back pain.

All study subjects answered a clinical and epidemiological questionnaire with data on age, gender, ethnicity, occupation, education, income, weight, height, smoking habits, comorbidities, physical examination, information regarding complaints, painful, and family history. All patients signed the form of free and informed consent approved by the local ethics committee.

The MRI scans of all patients were performed by two experienced radiologists. The DD was classified according to the classification of Pfirrmann *et al.* (2001), and only patients with Pfirrmann 3, 4, or 5 were included in the case group, once moderate and intense DD is evident.

Molecular analysis

Peripheral blood was collected from each patient and control in an EDTA-containing tube. Genomic DNA was extracted from peripheral blood of all study subjects according to the salting out method (Lahiri and Numberger, 1991).

Genotyping of the VDR *polymorphism*

The *FokI*/T2C/rs222857 polymorphism of the *VDR* gene was studied by restriction fragment length polymorphism–polymerase chain reaction (PCR) according to the protocol of Horst-Sikorska *et al.* (2007), with modifications. In general, the PCR procedure was carried out in a total volume of 25 µL reaction mixture containing 10X reaction buffer (500 mM KCl, 100 mM Tris–Cl; pH 8.3), 2.5 mM $MgCl_2$, 0.8 mM dNTP, 2.0 U Taq polymerase, and 50 nM of each primer (forward: AGC TGG CCC TGG CAC TGA CTC TGC TCT and reverse: ATG GAA ACA CCT TGC TTC TTC TCC CTC). The cycling profile consisted of denaturation at 95°C for 30 s, 60°C for 60 s for annealing temperature, and extension at 72°C for 30 s, except for the first cycle, when denaturation was extended to 5 min. The PCR product was digested with 5 U of FokI restriction enzyme (New England Biolabs, Ipswich, MA), and the reaction mixture was incubated at 65°C for 15 min. The digestion product was subjected to electrophoresis on a gel containing 2% agarose stained with ethidium bromide and visualized under ultraviolet light.

Using a DNA ladder of 50 base pairs (bp) as a reference, we identified the *Fok1* polymorphism genotypes: normal homozygote (TT) presented a unique fragment of 265 bp, heterozygote (TC) presented three fragments of 265, 196, and 69 bp, and mutant homozygote (CC) presented two fragments of 196 and 69 bp (Fig. 1).

Statistical analyses

Statistical analyses were carried out using SPSS for Windows 11.0 (SPSS, Inc., Chicago, IL). The chi-square test was

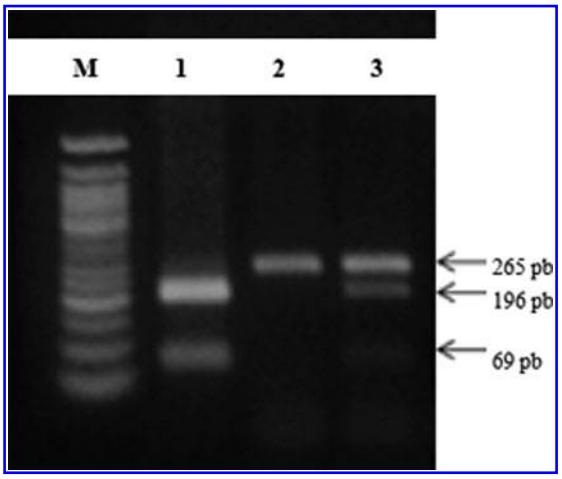

FIG. 1. The figure shows a 2% agarose gel representative of the VDR Fok1 polymorphism, where (M) is the DNA ladder 50pb, (1) is a mutated subject (CC), (2) is a wild homozygote (TT), and (3) is a heterozygous subject (CT).

used to compare allele and genotype frequencies between groups and to estimate the Hardy–Weinberg equilibrium. Genetic Power Calculator (Purcell *et al.*, 2003) was used to estimate the statistical power of the results concerning *Fok1* polymorphism data and showed at least 89% of power to detect the genetic effects regarding the association with DD for the allele frequencies and sample size in the present study. The odds ratio (OR) and range with 95% confidence interval (CI) were calculated for the presence of the reference genotype using a logistic regression model. All *p*-values were two-tailed, and 95% CIs were calculated. A *p*-value of <0.05 was considered statistically significant.

Results

Among the 121 subjects studied with DD, 46.3% (56/121) were male (mean age 46.0 ± 5.4 years) and 53.7% (65/121) were female (mean age 45.2 ± 5.9 years). Considering the control group, 26.7% (31/131) were male (mean age 33.8 ± 8.2 years) and 76.3% (100/131) were female (mean age 33.9 ± 8.1 years).

The genotype and allele distributions of the *Fok1* polymorphisms of the *VDR* gene in DD patients and controls are shown in Table 1.

Considering the genotype distribution in the DD patients, 44.6% (54/121) presented normal homozygote genotype (TT), 41.3% (50/121) presented a heterozygote genotype (TC), and 14.1% (17/121) presented a mutant homozygote genotype (CC). In the control group, the genotypes TT, TC, and CC were found in 57.2% (75/121), 35.2% (46/131), and 7.6% (10/131) of the subjects.

Regarding the allele frequencies, the wild allele T was found in 65.3% of the DD patients and in 74.8% of the control group; and the mutant allele C were observed in 34.7% of the DD patients and in 25.2% of the control group ($p = 0.025$; OR = 1.58, CI = 1.07–2.32).

Statistical analyses showed that the genotype distribution in DD and the control group for *FokI* polymorphism were in the Hardy–Weinberg equilibrium.

Discussion

The publication of the human genome sequence led to a great increase in the biomarker study field. The search for genetic associations with various diseases, including cancer, infertility, and other complex diseases, had great momentum to identify prognostic or preventive markers. Polymorphisms may serve as genetic markers, are responsible for human diversity, and can directly influence the risk factors associated with common diseases. Thus, the polymorphisms are key elements in the research and practice of human genetics (Kalichman and Hunter, 2008; Bag *et al.*, 2012).

Nowadays, polymorphisms are the basis for the attempt to provide personalized medicine based on genomics, based on the fact that if an individual carries polymorphic variants that increase or decrease the risk for common diseases in adulthood (such as coronary heart disease, cancer, diabetes, endometriosis, or DD) probably presents more complications after the surgery, or influence the effectiveness or safety of specific medications (Bag *et al.*, 2012).

Polymorphism refers to a variant of a particular gene sequence found in more than 1% of the general population. Single-nucleotide polymorphisms (SNPs) generally have two alleles corresponding to two different bases that occupy a particular position in the genome (locus). There are over 3 million SNPs documented and are found in virtually all genes, but only a minority results in changes in amino acids and lead to alteration of protein conformation. When this polymorphism results in a change of amino acid encoding a protein with altered function, these characteristics make them excellent markers to generate genetic maps such as those needed to evaluate the potential contribution of a given gene for a complex disorder. SNPs have been identified in genes responsible for metabolism, cell proliferation, transport, inflammatory response, immune response, and DNA repair that may be related to the development and progression of a disease and also in response to a specific treatment or preventive disease development (Kalichman and Hunter, 2008). There is not much information regarding how the polymorphisms affect vitamin D receptor transcription. The *VDR FokI* polymorphism in exon 2 leads to an alternative transcription initiation site, resulting in a VDR protein with the addition of three amino acids (Mory *et al.*, 2009; Vilarino *et al.*, 2011).

In this study, we hypothesized a possible relationship between *FokI* polymorphism of *VDR* gene and intervertebral DD. We found a significant difference in the frequencies of the polymorphism studied between DD patients and controls.

Previously, a series of studies have been conducted to evaluate the associations between *FokI* polymorphism of *VDR* gene and the risk of intervertebral DD but produced conflicting results. In 2003, Noponen-Hietala *et al.* (2003) studied *Fok*I polymorphism in 29 Finnish probands and 56 controls and a difference in the genotype and allele frequencies was not found. Eskola *et al.* (2010) evaluated 352 Danish children with early DD, and no statistical difference was found. Eser *et al.* (2010) investigated 300 young Turkish individuals regarding DD and herniation. An association was found in the patients having *VDR* gene TT (''T'' wild allele

Table 1. The Genotype and Allelic Distributions of the *FokI* Polymorphism of the *VDR* Gene in Intervertebral Disc Degeneration Patients and Controls

SNP VDR	*Population studied*	n	*Genotypes*			*Alleles*		p	*OR (95% CI)*	*HWE*
			n *(%)*	n *(%)*	n *(%)*	n *(%)*	n *(%)*			
			TT	TC	CC	T	C			
FokI	DD patients	121	54 (44.6)	50 (41.4)	17 (14.0)	158 (65.3)	84 (34.7)	0.025	1.58 (1.07–2.32)	0.624
rs2228570	Controls	131	75 (57.2)	46 (35.2)	10 (7.6)	196 (74.8)	66 (25.2)			0.737

CI, confidence interval; EHW, Hardy–Weinberg equilibrium; IDD, intervertebral disc degeneration; OR, odds ratio; SNP, single-nucleotide polymorphism.

and ''t'' mutant allele of *TaqI* polymorphism of *VDR* gene), Tt, FF (''F'' wild allele and ''f'' mutant allele of *FokI* polymorphism of *VDR* gene), and Ff genotypes with the protrusion type of disc herniation, whereas the patients having tt and ff genotypes were associated with extrusion/sequestration types of the disease. In addition, an association was observed between TT and FF genotypes of the *VDR* gene and mild forms of DD and also among tt, ff, and Ff genotypes and severe forms of the disease. Kelempisioti *et al.* (2011) investigated 538 young adults belonging to the 1986 Northern Finland Birth Cohort. The results disclosed that no association was found between DD and the *FokI* polymorphism.

The discrepant findings in the literature suggest genetic heterogeneity within the *VDR* gene in different diseases and populations, possibly due to divergent evolutionary lineages resulting in separate clusters of distinct geography (Vogel *et al.*, 2002). Consequently, the structure of linkage disequilibrium differs markedly across genomic regions and populations, and the extent of linkage disequilibrium is highly dependent on the population in which it is measured (Neale and Sham, 2004). Thus, the same allele may have different patterns of association with markers in different populations (Goldstein, 2001). Other aspects should be considered is the sample selection.

The first meta-analysis performed by Xu *et al.* (2012) included five studies that evaluated *FokI* polymorphism and DD (Noponen-Hietala *et al.*, 2003; Chen *et al.*, 2007; Eser *et al.*, 2010; Eskola *et al.*, 2010; Kelempisioti *et al.*, 2011). No significant association was found considering the polymorphism and the development of DD. However, the authors point to the fact that since potential confounders could not be ruled out completely, further studies are needed to confirm these results.

In conclusion, our results suggest that, in the Brazilian population studied, intervertebral DD risk is associated with *FokI* polymorphism of the *VDR* gene. However, further studies on much larger samples are needed to evaluate whether or not this association is real.

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Author Disclosure Statement

The authors disclosed no conflict of interest.

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