Original Article

Association between single nucleotide polymorphism in collagen IX and intervertebral disc disease in the Indian population

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Abstract

Background: Symptomatic intervertebral disc degeneration is being recently reported in younger population, questions the basis of its degenerative etiology. Latest evidences show that genetics play a significant role. Collagen IX, an important constituent of disc, is found to be altered in genetically predisposed individuals. Mutations have been reported in *COL9A2* and *COL9A3* genes, which encode Collagen IX, in Finnish and various other populations. The purpose of the present study is to test the significance of these genes in the Indian population.

Materials and Methods: One hundred proven cases of intervertebral disc disease (IDD) of various regions of spine were selected for the study, along with matched controls. They were tested for the above mentioned alleles by allelic discrimination method with real-time polymerase chain reaction (PCR) study after isolation of DNA from blood sample. Each blood sample was classified into one of the three types – homozygous, heterozygous, and wild (normal) type allele – separately for *COL9A2* and *COL9A3* genes.

Results: Homozygosity for *COL9A2* allelic variation was associated with 100% occurrence of the disease. Heterozygous allele of *COL9A2* was significantly higher in the study group (42%) as compared to the control group (17%). In contrast, allelic variation in *COL9A3* gene was found to have no significant correlation with disc disease. There was no single patient with homozygous allelic variation for *COL9A3*, suggesting predominance of *COL9A2* variation in the Indian population.

Conclusion: This candidate gene strategy approach adds considerably to our knowledge of genetic makeup of Indian populations in relation with disc disease. This study highlights importance of COL9A2 gene variation especially of homozygous variety in contrast to COL9A3 variation in causing disc disease in Indian population.

Key words: Allelic discrimination, *COL9A2* and *COL9A3* genes, intervertebral disc disease, real time PCR

Introduction

Intervertebral disc disease (IDD) is usually produced on compression of nerve root by herniated disc and is characterized by radiating pain along the course of nerve root in affected dermatome with or without neurological weakness in the corresponding muscles, though it even includes symptomatic disc degeneration without sciatica. Disc degeneration is usually an asymptomatic phenomenon associated with loss of proteoglycans and water content from disc, leading to loss of disc height. Degenerated disc usually losses its shock absorbing function and becomes fibrotic; fissure appears inside the disc with disorganization of annulus fibrosus. Sometimes, this degeneration can become symptomatic and culminates into disc disease. Thus, disc degeneration and disc disease are interlinked phenomena.[1] Etiology of disc disease consists of various environmental and constitutional risk factors, such as obesity, smoking, occupation, tall stature, lifting heavy loads, and mental distress, along with aging being most related.[2-4] Symptomatic intervertebral disc degeneration is being commonly seen in younger population,[5] questions the basis of its degenerative etiology. Additionally, it has been found to be more common in certain families.[6-9] Conventionally, it has been considered to be occurring due to aging-related degenerative changes. With increased use of magnetic resonance imaging (MRI) for spine screening in backache, we see large numbers of asymptomatic intervertebral disc degenerative changes on a frequent basis.

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These findings further raised doubt regarding the etiology of disc disease. But recent evidences are showing that other constitutional factors like genetics do play a significant role in its etiology, along with aging and environmental factors.

Various studies all over the world have emphasized the importance of genetic factor in causation of disc disease, especially a study conducted in Finnish population.[10] Collagen IX is a heterotrimeric protein composed of three genetically different α chains, α1 (IX), α2 (IX), and α3 (IX), encoded by the *COL9A1*, *COL9A2*, and *COL9A3* genes, respectively.[11,12] A Glutamine326 Typtophan change in the α2 chain and an Arginine103 Tryptophan change in the α3 chain of collagen IX have been identified in Finnish sciatica patients.[10,13] Mutations in genes encoding for matrix metalloproteinase-3 and vitamin D receptor genes have also been reported to be associated with disc degeneration.[14,15]

Alteration in collagen IX by substitution of Glutamine and Arginine by tryptophan can cause an alteration in the collagen properties of intervertebral disc. Tryptophan is relatively rare in collagen, and being hydrophobic it can cause alteration in collagen triple helix, as well as interfere with the interaction between collagens IX and II or prevent the action of lysyl oxidase, which catalyzes cross-link formation, in turn leading to disc disease.[16]

The present study was conducted to evaluate the association between cases with IDD and controls without IDD for allelic variation of *COL9A2* and *COL9A3* genes with the help of real-time polymerase chain reaction (PCR) study [Figure 1] in the Indian population.

Materials and Methods

Informed written and valid consent was obtained from all cases and controls participating in the study after explaining nature of the test and study design. Institutional Ethics Committee clearance regarding the study was obtained, and blood samples were collected for genetic tests after informed consent was obtained from the individuals.

Selection of study group

One hundred cases of IDD of various regions of the spine including cervical, dorsal, and lumbosacral region, who presented to us with clinical evidence of disc disease at the time of study, were selected. We had 65 males and 35 females with an average age of 41.98 years and the most common level of herniation being L4–L5 [Figure 2]. The Inclusion criteria were Age group: 15–60 years, Occupation consisting of sedentary lifestyle, Clinical evidence of disc disease with pain on visual analogue scale more than 20 and duration of pain along with radiculopathy more than 3 months, MRI obtained during symptomatic period with 1.5 T using T2W (TR/TE of 4000/90 ms) and T1W (TR/TE of 600/10 ms) sequences showing evidence of disc degeneration dark nucleus pulposus after comparing its signal intensity with adjacent CSF, i.e. grade 2 and above of Schneiderman's classification,[17] with obvious disc bulge compromising spinal canal.

The exclusion criteria were age group more than 60 years, heavy occupations like manual laborers or persons dealing with vibratory tools, body mass index more than 30, smokers, and individuals with history of psychiatric illness were excluded from the study.

We tried to compare disease severity in the study group by using visual analogue score (1–100) for radiculopathy. We also used radiological classification for disc degeneration severity, i.e. Schneiderman's classification for MRI which is: grade 1 (normal), normal height and signal intensity; grade 2 (intermediate), speckled pattern or heterogeneous, decreased signal intensity; grade 3 (marked), diffuse loss of signal; and grade 4 (absent), signal void.

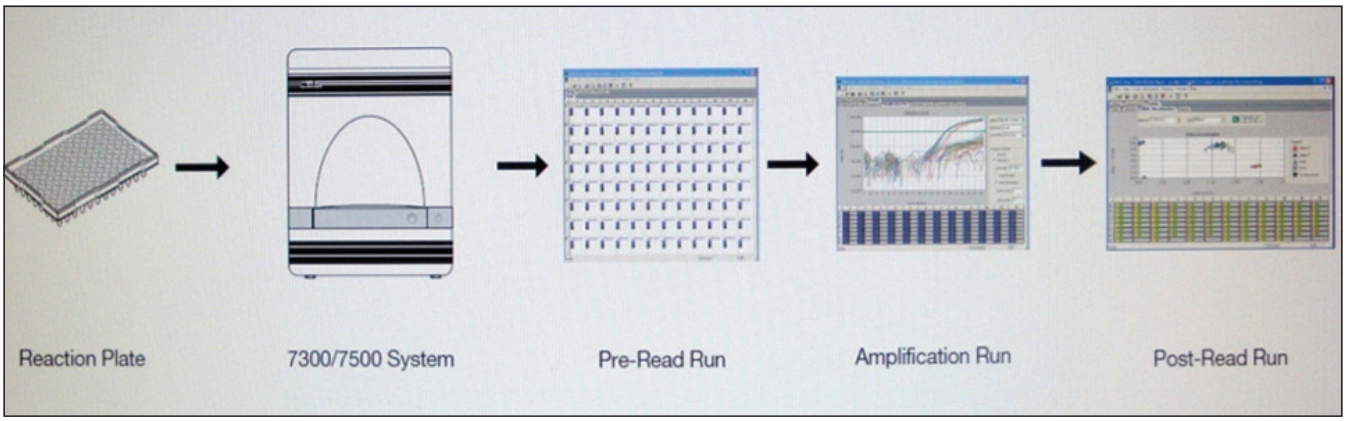

Figure 1: Diagrammatic representation of real-time PCR

One hundred controls including 67 males and 33 females, age-matched to the study group and without any clinical and radiological evidence (as mentioned for cases) of disc degeneration (asymptomatic), were selected for the study from individuals coming to hospital for unassociated problems like fractures, with an average age of 39.85 years.

Under aseptic conditions, 2 ml of venous blood was collected in ethylenediaminetetraacetic acid (EDTA) bulb and stored at -20°C. DNA was isolated from the blood sample with a DNA purification kit, which provides fast and easy method of purification of total DNA for reliable application in further PCR study.

Use of allelic discrimination method

An Allelic Discrimination (AD) assay[18] is a multiplexed (more than one primer/probe pair per reaction) endpoint (data are collected at the end of the PCR process) assay that detects variants of a single nucleic acid sequence.

Real-time PCR system uses fluorescent-based PCR chemistries for qualitative detection of nucleic acid sequences [Figure 1]. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence. The actual quantity of target sequence is not determined. For each sample in an AD assay, a unique pair of fluorescent dye detectors is used, for example, two TaqMan® MGB probes that target an SNP site. Thus, AD assay was performed after isolation of the DNA sample and it consisted of the following:

- A pre-read run on an AD plate document to determine the baseline fluorescence associated with primers and probes before amplification.
- An amplification run using an Absolute Quantification (AQ) plate document to generate real-time PCR data, which were used to analyze and troubleshoot the PCR data for the AD assay.
- A post-read run using the original AD plate document, which automatically subtracted the baseline fluorescence determined during the pre-read run, then assigned allele calls (automatically or manually) using the amplified data, and later data were analyzed.

AD assay classifies unknown samples as:

- normal homozygote – samples having both alleles normal, i.e. wild type;
- abnormal homozygote – samples having both alleles abnormal, i.e. homozygous for *COL9A2/3* variation;
- heterozygote – samples having normal allele as well as allelic variant of *COL9A2/3*.

Thus, every blood sample was classified into one of the three types, i.e. homozygous, heterozygous, and wild type (normal type allele), each for *COL9A2* and *COL9A3* gene.

We tried to include all levels of IDD consisting of cervical, thoracic, and lumbosacral region [Figure 2].

Results

Profile of *COL9A2*

Tables 1 and 2 reveal that 57.0% of the cases in the study group had an allelic variant of *COL9A2* which was significantly more as compared to 17.0% among the control group. Not a single patient had a homozygous variant of *COL9A2* in the control group, but in the study group 15.0% cases had a homozygous variant, with mean age of 42 years [Figures 3 and 4]. 42.0% of the total cases had heterozygous variant of *COL9A2* in the study group, with a mean age of 41.75 years, which was significantly more than 17.0%

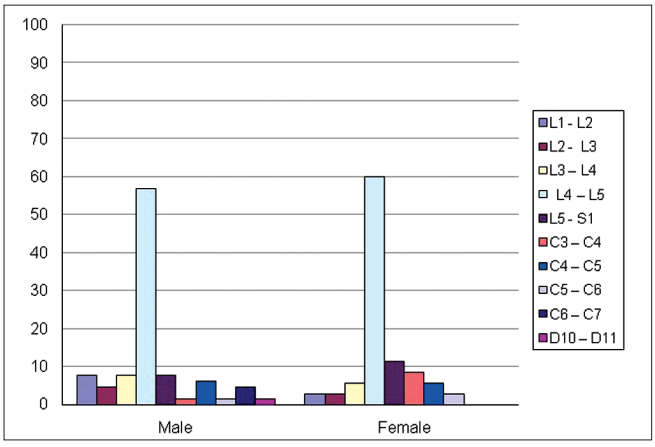

Figure 2: A bar diagram showing association of study group with levels of disc herniation

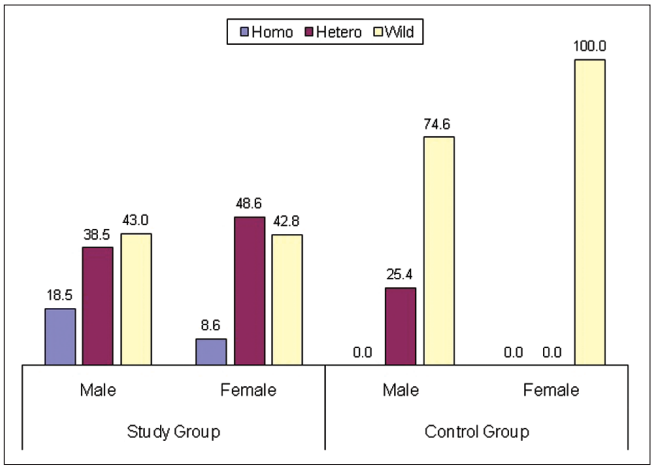

Figure 3: A bar diagram showing profile of *COL9A2*

Table 1: Profile of total *COL9A2*

COL9A2 alleles	Study group (*n*=100)		Control group (*n*=100)	
	No.	%	No	%
Homozygous	15*	15.0	-	-
Heterozygous	42*	42.0	17	17.0
Wild	43	43.0	83	83.0

By χ^2 test. *P<0.05 significant

Table 2: Profile of total *COL9A2* (sex distribution)

COL9A2 alleles	Study group (*n*=100)				Control group (*n*=100)			
	Male (*n*=65)		Female (*n*=35)		Male (*n*=67)		Female (n=33)	
	No.	%	No.	%	No.	%	No.	%
Homozygous	12	18.5	03	08.6	-	-	-	-
Heterozygous	25	38.5	17	48.6	17	25.4	-	-
Wild	28	43.0*	15	42.8*	50	74.6	33	100

By χ^2 test. *P<0.05 significant

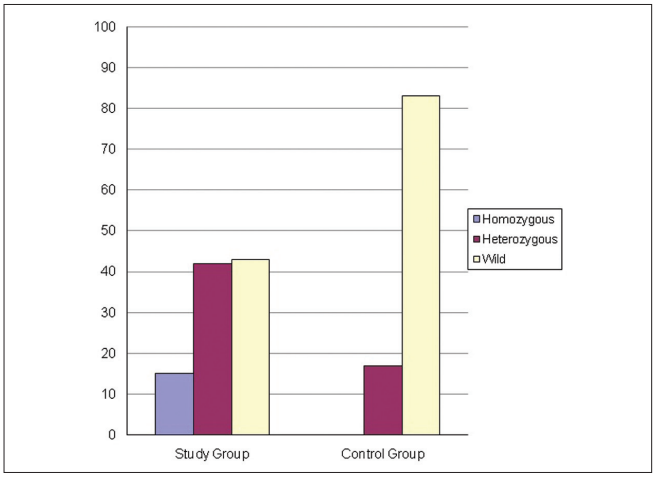

Figure 4: A bar diagram showing total profile of *COL9A2*

heterozygous variation among the control group. Forty three cases with a mean age of 42.19 years and 83 controls did not have allelic variation in *COL9A2*, i.e. had wild alleles.

Profile of *COL9A3*

According to Tables 3 and 4, no one had homozygous allelic variation for *COL9A3* in our study or control group. 5.0% of the total cases in the study group had a heterozygous allelic variant which was the same as compared to 7.0% among the control group and the difference was not statistically significant. 95 of cases and 93 of controls did not have allelic variation in *COL9A3*, i.e. had wild alleles [Figure 5 and 6]. Note that none of our cases who had allelic variation for *COL9A2* overlapped with one who had allelic variation for *COL9A3*.

Diffrence in median VAS Score at P=0.485 and radiological severity of disc disease at P=0.814 among the allelic variants of *COL9A2*, i.e. homozygous/heterozygous or normal, was found to be statistically insignificant [Table 5].

Table 3: Total profile of *COL9A3*

COL9A3 alleles	Study group (*n*=100)		Control group (*n*=100)	
	No.	%	No.	%
Homozygous	-	-	-	-
Heterozygous	05	05.0	07	07.0
Wild	95	95.0	93	93.0

By χ^2 test. P<0.05 not significant

Table 4: Total profile of *COL9A3* (sex distribution)

COL9A3 alleles	Study group (*n*=100)				Control group (*n*=100)			
	Male (*n*=65)		Female (*n*=35)		Male (*n*=67)		Female (*n*=33)	
	No.	%	No.	%	No.	%	No.	%
Homozygous	-	-	-	-	-	-	-	-
Heterozygous	03	04.6	02	05.7	07	10.4	-	-
Wild	62	95.4	33	94.3	60	89.6	33	100

By χ^2 test. *P<0.05 not significant

Difference in patients with neural deficit among different allelic variants groups was also not statistically significant. Hence, case groups with different allelic variants were comparable in terms of disease severity.

Discussion

As we know, IDD is not an isolated entity but is a continuum of serial changes which finally culminate into symptomatic disease. It is very interesting to find that radiological severity of the disease may not correlate clinically with disease manifestation, as other factors like nerve root inflammation due to disc are supposed to be important in the physical manifestations of radiculopathy.[19] Thus, it is very difficult to define disc disease for the study purpose, considering the fact of complex gene–environment interaction occurring simultaneously. So, we have considered both radiological and clinical criteria for inclusion of cases in the study group.

In our study, both study and control groups were comparable in terms of age, sex, and occupation. Allelic variant of *COL9A2* gene was found in 57% of the study population as compared to only 17% of the control population. This difference was highly statistically significant at a P value of <0.005. Thus, presence of *COL9A2* allelic variation was strongly associated with IDD, especially of homozygous variety. We had 15 subjects in the study group having homozygous variety of *COL9A2* allelic variation and all of them had disc disease as shown in Figures 3 and 4. Hence, homozygous variety was found to be associated with 100% occurrence of the disease. Though heterozygous variety of *COL9A2* was present in both study and control groups, relative frequency of the disease was much higher in the study group (42%) as compared to the control group (17%). This difference was statistically significant with a P value of <0.05. Thus, the overall frequency of *COL9A2*

Table 5: Comparison of severity of the disease with allelic variation in *COL9A2* gene

	Homozygous	Heterozygous	Normal	*P* value	Significance
Number	15	42	43	—	—
VAS score (median/IQ range)	70 (70–72)	72 (68–72)	70 (70–74)	0.485*	Not significant
Neurological deficit (No. of cases)	1 (6.7%)	2 (4.8%)	3 (7.0%)	0.905**	Not significant
Radiological grading (median/IQ range)	3 (3–4)	4 (3–4)	4 (3–4)	0.814*	Not significant

*Kruskal–Wallis test/**χ^2 test). *P*<0.05 significant

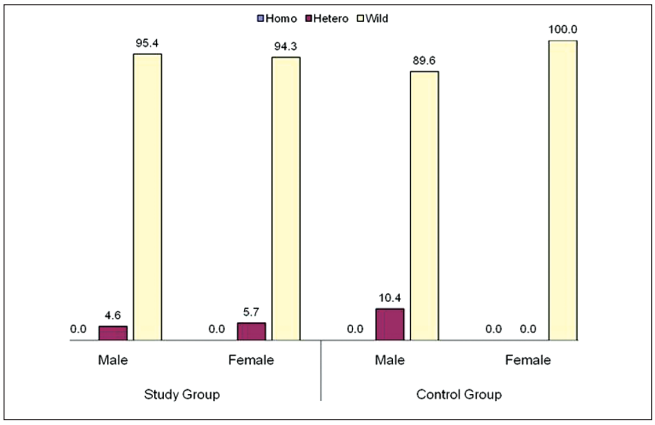

Figure 5: A bar diagram showing profile of *COL9A3*

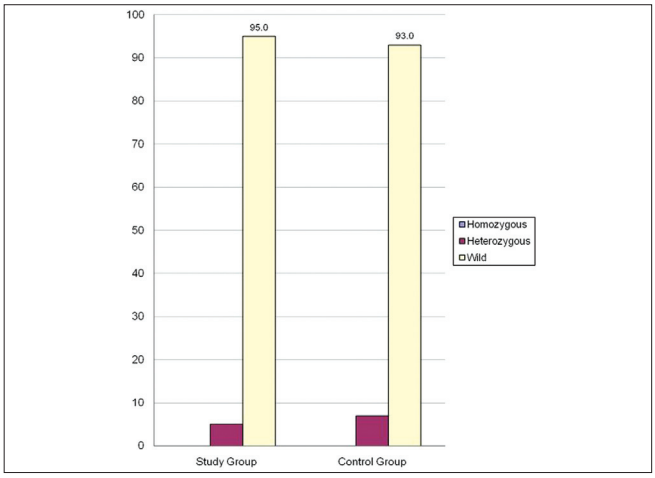

Figure 6: A bar diagram showing total profile of *COL9A3*

allelic variation was much higher in the study group as compared to the control group; hence, it is likely to play a significant role in the Indian population with respect to the occurrence of IDD. Remaining 43% study subjects showed no allelic variation, i.e. presence of wild allele. It means that in this particular group, no mutation (SNP) was found. So, the abovementioned genetic factor did not have any significant role in determining occurrence of disc disease in this group. Other environmental (physical) factor or an unknown genetic factor may be responsible for the occurrence of disc disease in them. This will require genome wide scan, which was not possible due to cost constraints. In contrast, heterozygous allelic variation of *COL9A3* gene, i.e. Trp3, was present in only 5% of the study group and 7% of the control population as shown in Figures 5 and 6. No one in either study or control group was found to have homozygosity for *COL9A3* variation. Thus, the presence of heterozygous *COL9A3* allelic variation was not associated with the occurrence of IDD at a *P* value of <0.005 in the Indian population. Homozygosity for *COL9A3* variation was absent in our study, so the relation of it with IDD cannot be determined. This was similar to a study conducted by Jim *et al.*[20] in the Chinese population in which Trp2 allele of *COL9A2* was found in 20% of the study population and Trp3 allele of *COL9A3* was absent. Contrary to the expectation, we found no significant difference in disease severity both radiologically and clinically in homozygous as well as heterozygous groups of *COL9A2*. We had six patients with neurological findings and all of them had grade 4 MRC (Medical research council)power in the affected muscle group corresponding to root, along with sensory loss in involved dermatome. Difference in patients with neural deficit among the different allelic variants was not found to be statistically significant. This probably indicates that these genes are more responsible for determining occurrence of disease rather than severity of disease, as severity of disease is more likely to be determined by complex gene–environment interaction. Further long term followup for the same is needed. We did not study allelic variation with disease severity in *COL9A3* as the sample size of cases tested positive for *COL9A3* was grossly inadequate.

Please note that it is very difficult to show any relation between occurrences of allelic variation and age group, level of herniation, occupation, or particular ethnicity (since India has multiethnic population), as it can be observed that disease load of IDD is distributed unequally among these age groups, occupations, or levels of herniation. Moreover, very large sample size of both study and control groups will be required for the same; otherwise, it can be spuriously labeled to particular level of herniation, age, or occupation.

A study conducted in Finnish population[10] showed that Trp2, i.e. *COL9A2* allele, was contributory in disc disease and it also included linkage analysis of family members. All members who had inherited the Trp2 allele in these families had IDD. *COL9A2* polymorphism was found only in a small percentage of Finnish population, but all individuals with this allele had disc degenerative disorders, suggesting that it is associated with a dominantly inherited disease. In our study, as the allelic variation of *COL9A2*

was also present in the control population, the disease may or may not be associated with dominant inheritance considering the fact that in the control population IDD may manifest in later part of life. Annunen[13] used conformation sensitive gel electrophoresis to analyze, whereas we used allelic discrimination with absolute quantification method for detection of Trp2 allele. They also found that presence of at least one Trp3 allele increases the risk of Lumbar disc disease about threefold. In contrast, we found that allelic variation of *COL9A3* is rare in the Indian population and *COL9A2* variation is present in comparatively larger frequency (57%) as compared to the Finnish population (3.82%). We did not consider linkage analysis in our study due to cost and time constraints. Solovieva *et al*.[21] tried to study gene–environment interaction and found increased incidence of lumbar disc degeneration for mutation of *COL9A3* gene associated with obesity. In our study, we tried to analyze gene phenotype (disc disease) relation by excluding patients with confounding factors like heavy occupation from the study group. Solovieva *et al*.[22,23] studied the interleukin-1 (IL-1) gene family and identified variation in IL1A and IL1B to be associated with low back pain and disc degeneration, suggesting that the effect of the *COL9A3* gene polymorphism on disc degeneration might be modified by the IL1B gene polymorphism. Variation in the abovementioned gene locus was not tested in our study and should be considered in future in the Indian context.

It can be seen that relative importance of these candidate genes varies in different geographic areas of the world, suggesting different gene pools in the world. Hence, coordination is needed among different countries for further understanding the importance of these set of genes with respect to region. However, necessity of random gene search cannot be overemphasized.

In conclusion, it is seen that *COL9A2* allelic variation, especially homozygous variety, is associated with occurrence of disc disease. So, presence of homozygous *COL9A2* variation can be considered as a genetic marker of disc disease in the Indian population. *COL9A3* is rare in the Indian population and is not found to be associated with disc disease. Thus, *COL9A2* plays a significant role in determining occurrence rather than severity of disc disease in the Indian population. In future, type IX collagen gene may be a good candidate for gene transfer and for modification in strategies to delay or prevent disc degeneration in susceptible populations.[24]

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