Al Ameen J Med Sci 2023;16(3):203-209 ● US National Library of Medicine enlisted journal ● ISSN 0974-1143

ORIGINAL ARTICLE CODEN: AAJMBG

Relation between TRP2 allele in COL9A2 gene and lumber disc degeneration in a subset of North Indian population

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Received: 15th March 2022; ***Accepted:*** 25th April 2023; ***Published:*** 01st July 2023

Abstract: *Introduction:* Lumbar disc prolapse is a disease in which the inner segment of disc ruptures from the outer segment. The disc prolapse starts as a result of lumbar disc degeneration (LDD). Various genetic factors were identified predisposing to higher risk of developing LDD. COL9A2 polymorphism is assuming a major role in the etiology of lumbar disc prolapse. *Aim:* This study was aimed to find the distribution of this polymorphism in the north Indian population and its true association with this disease. *Methods:* A total of 200 subjects (100 cases and 100 controls) were considered for this study. The cases were patients with complaint of low back pain who came to Rama Medical College Hospital and Research Centre, Kanpur between March 2018 and February 2020. The diagnosis of LDD was made by Magnetic resonance imaging (MRI) and clinical examination. The controls were volunteers (age matched), with no history of back pain or any other symptoms. Blood samples were collected from all subjects and DNA analysis was performed. Statistical analyses were analysed using SPSS version 22 (SPSS, USA).Odds Ratio (OR) with 95% Confidence Interval (CI) was applied to quantify the association of Trp2 allele to the Lumbar disc disease. Fisher's exact test was used to find statistical significance of contingency table counts, with a p-value<0.05 as significant. *Results:* Mean ages of cases and controls (in years) were 43±12.6 and 42.5±13.8 respectively. BMI(kg/m^2) of cases and controls were 23.71±4.27 and 22.93±3.87 respectively. The COL9A2 polymorphism was present in both cases and control. We found a 2.39 fold increase in the odds ratio of LDD when Trp2 allele present, OR 2.39 (1.06-5.38), p=0.03. *Conclusion:* The present study concluded that single nucleotide polymorphism in gene COL9A2 was found to be associated with the development of lumbar disc disease in the north Indian population.

Keywords: Disc prolapse, Back Pain, Polymorphism, Tingling, Numbness.

Introduction

A lumbar disc prolapsed is a condition of spine in which the inner component of intervertebral disc, the nucleus pulposus ruptures out from the outer segment, annulus pulposus [1]. The disc material fragment can press the nerve roots leading to lower back pain, tingling, numbness and muscle weakness in the lower body. The condition is also known as ruptured or herniated disc and commonly caused due to disc deterioration. The majority of the co-workers are agreed in favour of intervertebral disc degeneration as the main cause spinal disc herniation [2].

Lumbar disc degeneration (LDD) is a musculoskeletal disease of the lumbar spine in which age-related wear and tear on a spinal disc causes low back pain. It is a complex disease with genetic and environmental factors as important roles. There are multiple genes coding for collagen, matrix metalloproteinases, aggrecans, vitamin D receptor that play important role in the appearance of LDD [3]. Obesity, Physical load and smoking, are other factors which may contribute to the disease significantly [3].

The intervertebral disc has the features of a heterogeneous, cartilaginous structure which provide flexibility and help in load support in the spine region. Because of this reason the discs deteriorate far more rapidly than other tissues of the body. Due to extended

degeneration over a period of time, the blood vessels and the nerve fibres enter the avascular stage that causes pain [4]. Furthermore, this situation generates further disc deterioration by which it modifies the entire spinal mechanics and causes painful and disabling conditions. This reason for lumbar disc prolapsed or herniation of the nucleus pulposus is because of the defective annulus fibrosus [4]. The connection of this disease with other associated factors such as height, weight, and molecular involvement is still unexposed [5].

The reason for disc deterioration can be due to abnormal mechanical or chemical factors within the intervertebral disc. These agents, mechanical as well as chemical, can change the disc composition, structure, and properties [6]. The intervertebral disc is a specific type of fibrocartilaginous tissue which consists of an external layer known as the annulus fibrosis, and the internal layer known as nucleus pulposus. The annulus fibrosus has ring-like structure comprising for thick collagen I fibers. The nucleus pulposus has collagen II fibers (about 15-20% of its dry weight) and contains two other ligament collagens, types IX and XI as minor component. [7-8].

The two layers contain moderate quantities of collagen VI and minor of collagens III and V all through the circle. Proteoglycans account for around half of the dry weight of nucleus pulposus. Collagens give tensile strength to the disc and the proteoglycans give the tissue protection from compression forces. Collagen IX is a basic protein which connects different types of collagens together. Previous studies show that collagen gene polymorphisms are associated with risk of LDD [9]. COL9A2 polymorphism may be major role in the lumbar disc disease. Other factors like lifting heavy loads have been also related to the development of vertebral disc disease. Several other genes are found to be involved in the pathogenesis of intervertebral disc disease. Gene coding polymorphism for interleukins, vitamin D receptor and matrix metalloproteinases have been found to be related with LDD [10].

The mutations in COL9A2 gene have been found in Finnish and other populations [11]. Another study was conducted for genetic associations with collagen IX genes for disc diseases in the Indian population [10]. The most common age of presentation was 31-40 years (33.33%) and the incidence of lumbar disc prolapse above L4 was 13.3%. The incidence of lumbar disc prolapse was more common in people from rural area, moderate and heavy workers and vehicle drivers on bad roads [8].

They have done study specifically on workers on road.The objective of the study is to analyse the significance of single nucleotide polymorphism in genes (COL9A2) coding for one of the collagen fiber components in the north Indian population to confirm its true associations with this disease. In our study we have conducted in general patients coming in hospital due to pain, occupation not specified. This type of genetic studies in north Indian population was not conducted earlier.

Material and Methods

The study was approved by the ethical committee (ethics approval no: RU1601134) and informed consent was taken from all subjects. A total of 200 subjects (100 cases and 100 controls) were considered for this study. The duration of study was between March 2018 and February 2020 and type of study was case-control study. The cases in the study were the patients with symptoms of low back pain coming to Rama Medical College Hospital & Research Centre, Kanpur. The volunteers from the general population serve as control that were age and weight matched and with no history of back pain or any other symptom.

The sample size was calculated by following formula:

$$n = \frac{z^2 \times p \times q}{e^2}$$

Where,
n = number of subjects
p = expected prevalence
z = standard deviate (1.96 for 95 % confidence interval)
q = 1-p
e = error margin (10% for the study)

Different studies [12-15] have shown that the disc prolapse prevalence range from 27- 84%.

To get best possible number of participants in the study, the p value was taken as 0.5 leading to the value of q as 0.5. Putting these values in the formula, the value of n comes as 96. So total of subjects finally selected for this study comes as 200.

Inclusion criteria:

1. Age 18-60 years
2. Occupational not involving rigorous activity
3. Pain score more than 3 scores
4. Failed conservative management for a period of at least 3 months
5. MRI sequences with evidence of disc prolapse /extrusion/sequestration

Exclusion criteria:

1. Age > 60 years
2. Occupational like manual labourers lifting heavy weights or persons dealing with vibratory tools.
3. Body mass index (BMI) more than 30
4. Smokers
5. Spine deformity, metabolic bone disease
6. Spinal infection or tumors.

The imaging method, signs and symptoms were applied to diagnose lumbar disc disease. Lumbar disc degeneration grading was done by Schneiderman's classification of MRI (schneiderman et al. 1987 [16]).

Grade	Description
Normal	No signal changes
1	Slight decrease in nucleus Pulposus signal intensity
2	Hypointense nucleus pulposus with normal disc height
3	Hypointense nucleus pulposus with narrowing disc space

Genetic and molecular analysis: A blood sample from veins (2mL) was taken from all subjects in EDTA tube and stored in deep refrigerator. DNA genome was isolated with the help of Qiagen DNA mini Blood Kit. Gene polymorphism was seen by primers and the PCR method in COL9A2 gene on chromosome 1 (Trp2 allele). Primers of COL9A2 gene were designed with particular specification by Chromous Biotech Pvt. Ltd, Bengaluru.

Primer Sequence were:

Forward primer:

S.NO.	Primers Sequences	Tm(℃)
1	F5'- TGGATCTCAGTTTCCCTACCTG-3'	55.9
2	R5'-CAAGAGGTGGTGATTGAGCAAGAGC-3'	55.9

Reverse Primer:

S.NO.	Primers Sequences	Tm(℃)
1	F3-ACCTAGAGTCAAAGGGATGGAC-5'	55.9
2	R5-GTTCTCCACCACTAACTCGAACTCG-3'	55.9

Isolated DNA was amplified with PCR. The cycling condition of PCR was conducted for gene amplification.

PCR conditions:

- Initial denaturation: 950c-3mins
- Denaturation: 950c-30secs
- Annealing: 51oc-30secs
- Extension: 720c-50secs
- Cycling condition: 37 cycles
- Final extension: 720c-5mins
- Hold at 40c After amplification, out of 80 µl PCR product of study sample, 20 µl PCR product and 20 µl of PCR product of control were run with 1% agarose gel containing Ethidium bromide and observed under UV light in gel documentation system (Bio-Rad)

The products of PCR were resolved with 1% agarose gel electrophoresis. The resulting DNA bands were then assessed under UV light.

Data collection and statistical analyses: Odds Ratio (OR) with 95% Confidence Interval (CI) was applied to quantify the association of Trp2 allele to the Lumbar disc disease. Fisher's exact test was used to find statistical significance of contingency table counts, with a p-value<0.05 as significant. Statistical analyses were analysed using SPSS version 22 (SPSS, USA).

Results

Table 1 provides the particulars of enrolled subjects in the study. Mean age of cases was 43 years (30–70 years) and of controls was 42.5 years (32–66 years). There was no significant difference between distribution of sex and age among cases and controls. Disc prolapse was seen in most of the patients at L5-S1 levels.

Table-1: Baseline data of participants

		Cases (n=100)	Controls (n=100)	Chi square	p value
Age(years)		43±12.6	42.5±13.8		0.080
Gender	Male	66	66		
	Female	34	34	1.43	0.063
BMI (kg/m^2)		23.71±4.27	22.93±3.87		0.24
Smoking	Yes	25	21		
	No	75	79	0.45	0.5
Manual labourer	Yes	42	44		
	No	58	56	0.08	0.78

Majority of cases presented with pain in lower limb, back pain and numbness or tingling (Table 2, Fig.1 & Fig.2). There was no significant association between disc degeneration and lumbar injury (Table 3).

Table-2: Clinical findings in Cases (patients)

Clinical findings	Males	Females
PL+ PB +NT	40	18
PB + NT	15	10
PB + WL	11	06

Pain in lower limbs (PL), Backbone pain (PB); Weakness in limbs (WL), Numbness/tingling (NT)

Fig-1: Clinical findings in Male Cases (patients)

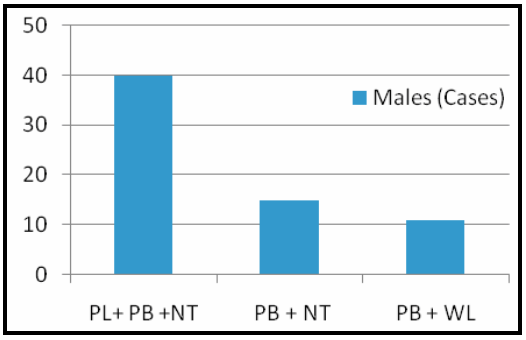

Fig-2: Clinical findings in Female Cases (patients)

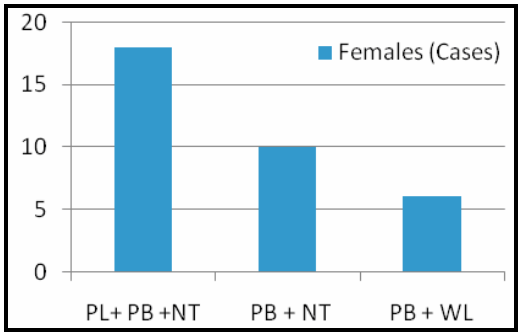

Table-3: MRI Changes showing lumbar injury in patients

		Cases (n=100)	Controls (n=100)	Chi square	p value
Schneiderman stage showing lumbar injury	Yes	12	7		
	No	88	93	1.45	0.23

Frequency of Trp2 allele: Fig.3 shows Trp2 allele in DNA bands of a subject. The Trp2 allele was found in both the cases and controls (Table 4). The Trp2 allele was positive in 31 participants (21 cases and 10 controls) OR

2.39 (1.06-5.38). A chi-square was used to examine the relation between Trp2 allele and LDD. The association between Trp2 and risk of LDD was significant, X^2 (1, N = 200) = 4.62, p = 0.03.

Fig-3: DNA bands visualized under ultraviolet light for Trp2 allele

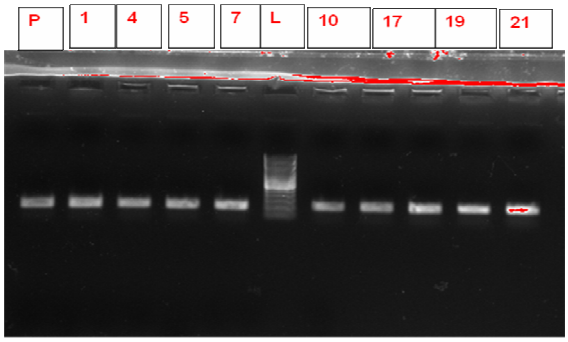

Table-4: Frequency of Trp2 allele in COL9A2 gene			
	Trp2 allele in COL9A2		
	Positive	Negative	Total
Case	21	79	100
Control	10	90	100
Total	31	169	

Table-5: Disc degeneration and Trp2 allele in patients					
	Total	Grade 2 or 3	Grade 4	Odds ratio	p value
Trp2+	21	8	13	3.72	0.04
Trp2-	79	55	24		

Odds ratio was calculated to find the association between Trp2 allele and severity of disc degeneration (Table 5). Patients with the Trp2 allele were found to have more severe disc degeneration, as revealed by MRI (odds ratio 3.72, p=0.04).

Discussion

Lumbar disc degeneration (LDD) is a disease caused by various environmental and genetic factors [17-18]. Lumbar Disc degeneration starts early in the life and is result of a various extrinsic and intrinsic factors, including the process of ageing. The aetiology of the disease is not clear even after extensive research in this field. The present study was done to analyse the significance of single nucleotide polymorphism in genes (COL9A2) coding for one of the collagen fiber components in the north Indian population to confirm its true association with this disease.

Three chains α1, α 2 and α 3 form a heterotrimer of Collagen IX. These chains are coded by the *COL9A1*, *COL9A2*, and *COL9A3* genes, respectively [19]. This is cross-linked to collagen II fibres [20] and serves as a connection between non-collagenous and collagenous material in tissues [21]. The role of this collagen type is shown for maintaining tissue integrity and supported by various studies [22-25].

Collagen IX is present in minor amount in annulus fibrosus as well as nucleus pulposus [26]. Gene polymorphisms or abundance of this collagen can be a predisposing factor for disc degeneration. Tryptophan amino acid is hydrophobic type which is rarely found in collagens. The presence of tryptophan can alter the function or structure of collagen IX present in the disc. The substitution of tryptophan in the α2 chain of collagen IX (Trp2 allele) can alter the mechanical integrity leading to disc degeneration over the years.

Annunen et al [27] found the association between the Trp2 allele and intervertebral disc disease. They found the Trp2 allele in 6 out of 157 individuals with cases of sciatica but in none of the 174 controls. Radiologically detectable intervertebral disc disease was present in 73% of the cases. Similar findings have been seen in a large population case-control study in the China [28].

They found Trp2 allele in 20% of the participating population. Individuals with Trp2 allele were found to be at great risk of developing annular tears and disc degeneration and severity of disc degeneration was directly associated with Trp2 allele. Higashino et al [29] performed a study lumbar discectomy patients in Japanese population and Trp2 allele was found in 21.3% of study population. They also found that Trp2 allele was an age-dependent risk factor and younger patients (<40 years), disc degeneration was more severe when Trp2 allele was present.

However, no significant association was found between collagen IX genotype and disc degeneration in patients over 40 years of age. In a study done on 105 patients of intervertebral disc disease in Greece, no Trp2 allele was found [30].

In a study done in German population, no significant association was seen between Trp2 allele and disc degeneration [31]. The different results of linkage between disc degeneration and Trp2 may be due to differences in the frequencies of allele and genotype between different ethnic groups.

Our study design consists of the few limitations. First, a very small sample size was used in the study. The participants selected from a single hospital, and may not be represented for all patients for north India having lumbar disc disease. So, subject selection bias is unavoidable. Secondly, there are various genes that contribute to the development of this condition and gene-gene interaction study should also be considered in future.

Conclusion

The present study concluded that single nucleotide polymorphism in gene COL9A2 was found to be associated with the development of lumbar disc prolapse in the north Indian population. This study gives a basic understanding of the distribution of this genotype in the north Indian population. Further studies in larger cohorts are required to confirm our results.

Acknowledgement

We would like to thank all the subjects who participated in this study.

Financial Support and sponsorship: Nil

Conflicts of interest: There are no conflicts of interest.

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Cite this article as: Ekramuddin, Tewari V, Kumar A and Das M. Relation between TRP2 allele in COL9A2 gene and lumber disc degeneration in a subset of North Indian population. *Al Ameen J Med Sci* 2023; 16(3): 203-209.

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