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Research Article

Detection of Vitamin D Receptor Gene Polymorphism in Degenerative Spinal Conditions among Sudanese Patients.

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Abstract:

Background:
Spinal Degenerative is common condition characterized by the breakdown of one or more of the disc that separate the bones of the spine causing low back pain. Degenerative disease of the lumbar spine is a significant cause of disability in the world; it encompasses conditions such as spondylosi, disc degeneration and lumbar spinal stenosis associated with a variety of clinical symptoms including lower extremity pain ,weakness, and low back pain (LBP) of varying levels of severity, lumbar degenerative spine disease can lead to a reduction in the quality of life.The objective of this study is to detect VDR gene rs731236 in degenerative spinal conditions among Sudanese patients.

Material and methods:
This was prospective cross sectional study, conducted at the research center of the National Center for Neurological Sciences (NCNS), Khartoum state, Sudan, during the period from May to July 2022. It is included all operated patients admitted to National Center for Neurological Sciences and were diagnosed with degenerative spinal conditions. DNA extracted from tissue according to the protocols adopted in NCNS molecular laboratory, PCR and Sanger sequencing for vitamin D receptor gene were done.

Results:
The findings of this study revealed, G>C rs376903517 was detected in 43% of the samples, A>C rs75590999 in 29%, moreover, each of deletion C and G>A was detected in 14% of the samples.

Conclusion:
G>C rs376903517 mutation was the most frequent among our patients in 43%; this mutation was predicted as disease causing.

Keywords: Degenerative spine, VDR, PCR, Sequencing.

Supplementary information The online version of this article (https://doi.org/xx.xxx/xxx.xx) contains supplementary material,which is available to autho-rized users.

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Introduction:

Degenerative spine is common condition characterized by the breakdown of one or more of the discs that separates the bones of the spine causing Low back pain. The degenerative processes involve the structural damage of the intervertebral disc and change in number and composition of cells. The main factor in the degeneration of the intervertebral disc is loss of proteoglycans. Degenerative changes of the intervertebral disc are connected to damage of nearby structure, such as ligaments, joints and vertebral muscles, this leads to functional changes and greater susceptibility to injuries. [1]

Degenerative disease of the lumbar spine is a significant cause of disability in the world; it encompasses conditions such as spondylosis, disc degeneration and lumbar spinal stenosis associated with a variety of clinical symptoms including lower extremity pain, weakness, and low back pain (LBP)of varying levels of severity, lumbar degenerative spine disease can lead to a reduction in the quality of life. [2] Several specific genetic markers have been linked to disc degeneration ,including alleles of the vitamin D receptor(VDR), tandem repeats within the aggrecan gene ,type IX collagen mutations and alleles of the matrix metalloproteinase-3(MMP-3)gene .in addition to direct gene effects ,environmental factors can interact synergistically to produce disc degeneration as shown by the combination of obesity and mutant type IX collagen gene ,which leads to a higher than –expected rate of severe disc degeneration in obese individuals .[3]

The VDR is a nuclear receptor that functions as a transcription factor, and most of its activities are regulated by its ligand, 1, 25$(OH)_2$ D, VDR belongs to the family of steroid receptors which include the adrenal steroid, sex hormone, thyroid hormone and retinoic acid receptor, and VDR is widely distributed among different cell line ages. Vitamin D plays an important role in calcium homeostasis and bone metabolism, with the capacity to modulate innate and adaptive immune function, cardiovascular function, and proliferation and differentiation of both normal and malignant.[4]

The gene encoding VDR is mapped to human chromosome 12q13-14 region and is spanned approximately 100kb long, changes in the sequence of the gene such as polymorphisms, may occur in the non coding region of the gene (introns) affecting the level of gene expression and thus protein levels, and the coding regions (exons) leading to changes in the sequence of protein genetic alterations in the VDR gene lead to significant gene activation defects affecting calcium metabolism , cell proliferation and immune function, which can be an origin of intervertebral disc disease (IDD). [5]

Several studies investigated the role of VDR polymorphisms in the susceptibility to common disease (such as osteoporosis, Osteoarthritis and of intervertebral disc disease), even if the real influence on VDR protein function and signaling in those pathologies is largely unknown. Human VDR covers at least 105kb, with an extensive promoter region capable of generating multiple tissue-specific transcripts. several biallelic polymorphic sites have been identified in the VDR sequence both in coding and non-coding region, the most known and studied are Bsml , Taq1, Apa1 and Fok1 .[6]

The effect of the Taq1 alleles in vitamin D receptor was assessed on the risk of developing degenerative disc disease in a southern Chinese population. The t allele of Taq1 in VDR gene was significantly associated with a high risk of degenerative disc disease and disc bulge developing. [7]

Vitamin D can influence sulphate metabolism which is important for sulphation of glycosaminoglycans (GAGs) during proteoglycan synthesis. Thus a hypothesis is that the polymorphisms affect receptor level and function leading to changes in the structural characteristic of the extracellular matrix in the intervertebral disc. [8]

Materials and Methods:

This was prospective cross sectional study, conducted at the research center of the National Center for Neurological Sciences (NCNS), Khartoum state, Sudan, during the period from May to July 2022. It is included all operated patients admitted to National Center for Neurological Sciences and were diagnosed with degenerative spinal conditions. DNA extracted from tissue according to protocols of molecular lab National Center for Neurological Sciences (NCNS), PCR for vitamin D receptor gene was done and Sanger sequencing.

The Data was collected using pre-designed structural questionnaire, the demographic and clinical data taken from register data base office, which included the following information (gender, age, occupation and radiological finding), molecular finding of VDR gene included (PCR and Sanger sequencing results). All these data analyzed by using computer Statistical Package for the Social Sciences (SPSS). Version 13.

The result was expressed as percentage, P value less than 0.05 was considered as statistically significant, the genetic data was analyzed using bioinformatics' programs (mutation taster, Ensemble and Bio-Edit). The tissue sample were collected in sterile containers, and then processed for DNA extraction.

DNA extraction:

The DNA was extracted from tissue according to protocols of molecular lab in National Center for Neurological Sciences (NCNS), the tissue was cut into

small pieces, then 800µl of STE buffer was added until homogeneous, the homogeneous mixture placed in eppendrof tube that contains 100µl of 10% SDS and 20 µl proteinase K, the tube incubated at 65 C^0 for overnight. Then after that, protein was Precipitated by adding 300µl of 6M NaCl and kept in the refrigerator at $4C^0$ for 15min, after incubation, the tubes centrifuged at 18000 rpm for 20min. Furthermore, 500µl of the supernatant transferred to a new eppendroff tube, following this, 350µl of 8M guanidine chloride and 150µl 0.49 M NH4 acetate was added, and then incubated at room temperature for 90 min, after incubation, 500µl pre-chilled chloroform was added, and then centrifuged at 12000 rpm for 5min. After that, the upper layer transferred to a new tube, and then 800µl of cold absolute ethanol was added and the tubes incubated at -20 °C for overnight, then centrifuged at 12000 rpm for 5 min. the pallet was washed with 400µl of 70% ethanol, then vortex the tubes and centrifuged at 7000 rpm for 5min, the supernatant was pour off and the pallet was allowed to drying. Finally, DNA was eluted in ddH_2O.

Polymerase chain reaction:

DNA was performed using polymerase chain reaction to amplify specific sequence of VDR gene. Primers were designed by using prime3 software, the forward primer for VDR gene was (5-CTGCCGTTGAGTGTCTGTGT-3), reveres primer was (5-TCGGCTAGCTTCTGGATGAT-3) with product size of 242bp. following the procedure of molecular lab, 14 µl double distilled water was placed in PCR tube, then 4 µl of master mix, 1µl of reverse primer and 1µl of forward primer and 2 µl from the extracted DNA was added than vortex. after that the PCR tube contain this mixture was placed in commercial thermal cycler (Swift Maxpro SWT-MXP-BLC-4) at following condition, Denaturation temperature 94^0C for 30seconds ,annealing temperature at 61^0C for 30 seconds and extension temperature at 72C for 30 seconds , the final elongation was adjusted for 5 minutes at 72^0C. PCR reaction was set at 35 cycles PCR products subjected to a garose gel electrophoresis, the gel was prepared by dissolving 0.7 gm agarose gel in a clean conical flask containing a mixture of 28ml of DW and7ml of TBE, then the flask put at $60c^0$ in microwave for 1 minute, before the gel cool down 0.9ul ethedium bromide was added and mixed then poured on casting tray and left to dry. Then after that, 4 µl from each PCR product and 1µl from 100 bp ladder were loaded in the wells of casting tray, then the running buffer was poured and ran at 150V for 16 minutes and the results were trans-illuminated with UV light and visualized through gel documented system. The rest of DNA products were sent for high-quality Sanger sequencing **to** Macrogen Europe lab at Amsterdam.

This study was approved by the ethical committee of National Center for Neurological Sciences and Faculty of Medical Laboratories Science, Al-Neelain University.

Results

Demographic results

The frequency of gender in degenerative spinal conditions showed that, male were15 constituted 71% while female were 6 constituted 29%. (Tables 1)

The most affected age group was more than 60 years which was detected in 29% of the patients, followed by the age group 51 – 60 years in 24%. (Tables 2)

According to occupation, Workers were detected in 33% of the patients, followed by house wife in 24%, employee in 19% and students in 14%. (Table 3)

Table (1): Distribution of degenerative spine conditions within to gender

Gender	Frequency	Percent (%)
Male	15	71%
Female	6	29%
Total	21	100%

Table (2): Distribution of degenerative spine conditions within the age groups

Age group (Years)	Frequency	Percent (%)
10 – 20	1	5%
21 – 30	2	9%
31 – 40	3	14%
41 – 50	4	19%
51 – 60	5	24%
More than 60	6	29%
Total	21	100%

Table (3) Distribution of degenerative spine conditions within occupations

Occupations	Frequency	Percent (%)
Worker	7	33 %
House Wife	6	29 %
Employee	5	24%
Student	3	14 %
Total	21	100 %

Molecular results

In this study, the gel electrophoreses of VDR gene was displayed in Figure1, and multiple alignment findings were displayed in figur2, in addition to this, the analysis of sequencing results of VDR gene showed that, G>C mutation was detected in 43% of the samples, A>C in 29% and deletion C in 14% While G>A in 14%. (Table 4)

By using multiple bioinformatics tools, on top of using Mutation tasting online program, (G>C) rs376903517 mutation was predicted as disease causing, the impact of this mutation on protein shows amino acid change at position R368P, in addition to, this mutation may change the splice site and the protein feature might be affected (H3K27me3). Figure 3.

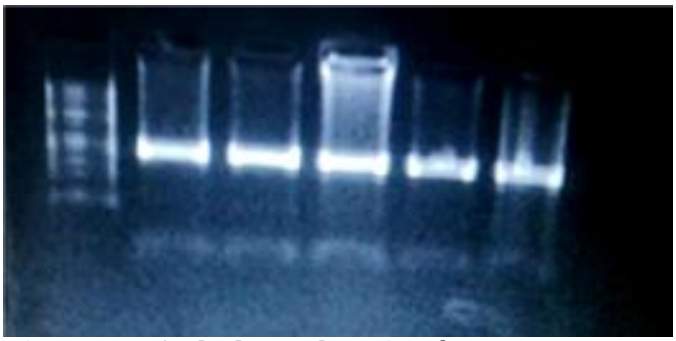

Figure (1): |gel electrophoresis of VDR gene (PCR product), lane 1: DNA ladder (100 bp), lane 2-6: VDR PCR product (band size=242 bp)

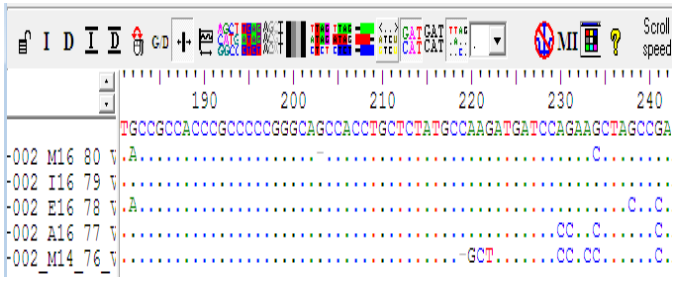

Figure (2): shows multiple sequence alignment using Bio-Edit soft ware of the samples with the reference sequence of VDR gene retrieved from Ensemble database.

Table (4): VDR gene mutation, frequency and percentage in degenerative spinal patients

Mutation	Frequency	Percent (%)
G>C	3	43%
A>C	2	29%
Deletion C	1	14%
G>A	1	14%
Total	7	100%

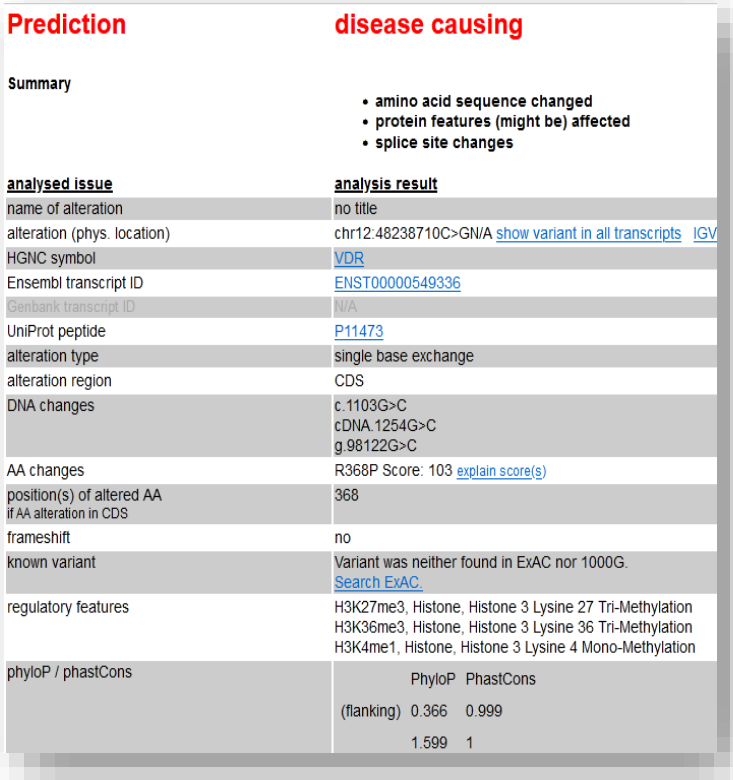

Figure (3): Using Mutation tasting online program, G>C mutation was predicted as disease causing mutation, the amino acid change at position R368P may change the splice site, and the protein feature might be affected (H3K27me3).

Discussion

Intervertebral disc prolaps can contribute to the development of low back pain (LBP) and acute lumbar radiculopathy associated with disc herniation. Back pain is associated with disc prolapsed and radial fissures especially when reaching the disc exterior, disc extrusion ,internal disc disruption , with inward collapse of the annulus and disc narrowing was also found to be associated with pain. [9]

Many Previous studies have revealed varying male : females ratios degenerative disc disease are more common in female than in male ,Female rats have an increased tendency to develop intervertebral discs degeneration in the lumbar spine after undergoing ovariectomy. Postmenopausal women have a significant tendency to develop more severe disc degeneration than their age matched men, where as in young cases, age-matched men are more susceptible to disc degeneration than premenopausal women; recent clinical studies have proposed that menopause may be an etiological factor in the development of lumbar disc degeneration. [10]

However after menopause which is around the age of 49-50 years , lumbar discs in females degenerate at a notably quicker rate than male lumbar disc . [11] Our result shows that degenerative disc disease according to gender 15 patient (71%) were male and 6 patient (29%) were female , in this study the male were affected more than female .

As people age, degeneration of intervertebral discs can occur at faster rates than for other tissues and is sometimes it presented on individuals as young as 11–16 years of age, Degenerative disc disease affects about 20% of people in their teens, showing mild signs of degeneration before their second decade of life. However, because the discs have yet to undergo progressive innervations, most cannot feel the pain and disabilities associated with degeneration until it propagates through to the later years of life. Therefore, this disease increases drastically with age, causing the discs of around 10% of 50-year-old population and 60% of 70-year-old population to become severely degenerated, significantly hindering daily activities. [12]

Additionally, various studies have shown that Disc diseases can begin as early as the second decade and increases linearly with age; hence at 70 years of age, (80%) of all lumbar discs are abnormal, moreover, by the age of 50 (85-95%) of e adults show evidence of IVD degeneration on autopsy. Matrix synthesis decreases steadily throughout life but occasionally increases again in old and severely disrupted discs the concentration of cells in the disc declines with age, especially in the annulus. They are subject to senescence, lose their ability to proliferate and may induce degeneration by decreased anabolism or increased catabolism. [9]

The most prevalent affected age in the current study, age group was 60 years and above and constituted nearly quarter of the patients. Further more in occupational spinal disease, there is a relatively accelerated and increased level of degeneration (especially in workers less than 45-50 years of age) , although prevalence normally increases with age . Disc, facet joints, and vertebrae are affected. [13] In the UK , approximately 20 million working days are lost each year because of LBP , and the latter accounts for 40% of the time lost due to industrial injury , occupational exposure has generally been assessed by categories of activity , for example , heavy lifting , frequent bending or broad occupational categories as truck driving . End points examined have ranged from symptomatic back pain to radiological change or surgical end points for disc prolapse. [14] our result show the most affected patients were worker.

Additionally, the direct effect of vitamin D on bone is contentious 1,25 (OH)2D has the capacity directly or indirectly to regulate the proliferation ,differentiation , and maturation of osteoblasts and osteoclasts , bone resorption , and mineralization . it appears that$1,25(OH)_2D$ via VDR up regulates the expression of genes encoding type 1 collagen, osteocalcin , and osteopontin that drive bone formation. In addition $1,25(OH)_2D$/VDR induces the expression of RANK ligand by osteoblasts , which in turn mediates differentiation and increased activity of osteoclasts, thus ensuring bone turnover. This sequence of metabolic processes suggests that 1,25(OH)2D has capacity to exert both anabolic and catabolic effects on bone.[9]

Vitamin D and its active metabolites participate in the processes of bone tissue mineralization, maintaining calcium homeostasis, and bone remodeling which is mediated through its receptor, the VDR receptor is expressed on the cell surfaces of the intestine, thyroid, and kidney and has a key role in calcium homeostasis. Its association with bone mineral density (BMD) Polymorphisms in the gene can occur in its coding or noncoding parts and lead to changes in the protein sequence; these can also affect the degree of gene expression. These include single nucleotide polymorphisms (SNPs) that can be identified with the appropriate restriction endonucleases such as ApaI and TaqI. [15]

Many mutations have been identified in the VDR that cause $1,25(OH)_2D$ resistance include nonsense mutations, insertions/substitutions, insertions/duplications, deletions and splice site mutations, and thereby cause hereditary vitamin D-resistant rickets(HVDRR). [16]

Our results providing vitamin D receptor gene polymorphism is linked to degenerative condition that might influence the proteoglycan function, the analysis of our sequencing, that found four single base exchanges, G>C rs376903517 mutation was predicted as disease causing, the impact of this mutation on protein shows amino acid change at position R368P, in addition to, this mutation may change the splice site and , Also A>Crs75590999 mutation was predicted as disease causing , the amino acid was changed from Isolusin at position 367 into Lysine, more over Deletion C mutation was predicted as disease causing, this mutation may change amino acid at position K382R. Furthermore G>A mutation was predicted as disease causing, shows amino acid change at position R368H. , the protein feature might be affected H3K27me3,histone, histone 3 lysine 27 tri-Methylation mutation within the regulatory and coding sequence(CDS) .

Conclusion:

In the conclusion VDR gene (G>C) rs376903517 polymorphism was detected and relation with degenerative spinal conditions among Sudanese patients and were found in 43 % of patients suggesting this mutation might be a risk factor for degenerative spinal conditions.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Author Contribution

All authors similarly contributed to this manuscript, covered wrote, corrected and authorized this manuscript.

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