Kocatepe Tıp Dergisi
Kocatepe Medical Journal
24:184-189 / Nisan 2023 Sayısı

ARAŞTIRMA YAZISI / RESEARCH ARTICLE

SPONDİLOEPİ (META) FİZYAL DİSPLAZİ ÖN TANILI BEŞ TÜRK ERKEK HASTADAKİ GENETİK ETYOLOJİNİN ARAŞTIRILMASI

INVESTIGATION OF GENETIC ETIOLOGY IN FIVE TURKISH MALE PATIENTS WITH PRE-DIAGNOSED SPONDYLOEPI (META) PHYSEAL DYSPLASIA

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ÖZET

AMAÇ: İskelet displazisi orantısız boy kısalığı ve çeşitli ortopedik komplikasyonlarla karakterize kompleks bir hastalık grubudur. X'e Bağlı Spondiloepifizyal Displazi Tarda, progresif spondiloepi(meta)fizyal displazi ve prematür osteoartritin eşlik ettiği X'e bağlı kalıtsal bir iskelet displazisidir. *Trafficking protein particle complex 2 (TRAPPC2)* geni bu hastalıkla ilişkilendirilmiştir. Progresif psödoromatoid displazi (PPRD), dirsek eklemlerinde genişleme ve artrit benzeri bulgular ile karakterize bir hastalıktır. Otozomal resesif kalıtımlı bir iskelet displazisi subgrubu olan bu hastalığa ise 6q21 kromozomal lokasyonunda bulunan *celluler communication network factor 6 (CCN6)* genindeki mutasyonlar neden olur. Bu çalışma ile X'e bağlı ya da otozomal resesif kalıtım paterni düşünülen, yıllardır tanı almamış beş erkek bireye tanı koyarak, olası tedavileri belirlemek ve prenatal preimplantasyon genetik test olanağı sunmak amaçlandı.

GEREÇ VE YÖNTEM: Bu çalışmaya, X'e bağlı veya otozomal resesif kalıtımlı iskelet displazisi olan beş erkek kardeş dahil edildi. Dört hastaya Whole Ekzom Sekanslama (WES) yapıldı. Bir hasta ve aynı aileden dört sağlıklı bireye ise Sanger sekanslama yapıldı.

BULGULAR: Etkilenen tüm kardeşlerde CCN6 geninde homozigot c.210C>A (p.Cys70Ter) ve c.302G>A (p.Gly101Glu) mutasyonları bulundu. Böylece, X'e bağlı resesif kalıtım paterni olma olasılığına rağmen WES sonrası otozomal resesif PPRD tanısı koyuldu.

SONUÇ: Bu çalışma normalde bir çocukluk çağı hastalığı olan Progresif psödoromatoid displazisi tanısı alan ortalama yaşı 54.6 olan en yaşlı hastaları sunmaktadır. p.Cys70Ter değişikliği Türk hastalarda en sık görülen patojenik varyanttır. Bu çalışma, Progresif psödoromatoid displazinin ortalama yaşam süresi üzerinde anlamlı bir etkisinin olmadığını göstermesi açısından da önemlidir. Aynı zamanda bu çalışma, bu hastalığın seyrini ve yaşam boyu eşlik edebilecek klinik bulguları göstermesi açısından da önemlidir.

ANAHTAR KELİMELER: Artropati, İlerleyici psödoromatoid displazi, *CCN6* geni

ABSTRACT

OBJECTIVE: Skeletal dysplasias is a complex disease group characterized by disproportionate short stature and various orthopedic complications. X-Linked Spondyloepiphyseal Dysplasia Tarda is an X-linked inherited skeletal dysplasia accompanied by progressive spondyloepi(meta)physeal dysplasia and premature osteoartritis. The gene related to the disorder is *trafficking protein particle complex 2 (TRAPPC2)*. Progressive pseudorheumatoid dysplasia (PPRD) is characterized by enlargement of the elbow joints and arthritis-like findings. It is an autosomal recessive subtype of skeletal dysplasia caused by mutations in *cellular communication network factor 6 (CCN6)* gene located on chrosomal region 6q21. In this study, it was aimed to diagnose five male individuals with an X-linked or autosomal recessive inheritance pattern, who have not been diagnosed for years, to identify possible treatments and to offer prenatal pmreimplantation genetic testing.

MATERIAL AND METHODS: Five male siblings with skeletal dysplasia with an uncertain inheritance either X-linked or autosomal recessive pattern were included in this study, Whole Exome Sequencing (WES) was applied to the four affected cases. Sanger Sequencing was performed in one affected case and four healthy individuals.

RESULTS: Homozygous c.210C>A (p.Cys70Ter) and homozygous c.302G>A (p.Gly101Glu) mutations in the *CCN6* gene were found in all affected siblings. Thus, the final diagnosis after WES was autosomal recessive PPRD despite the possibility of an X-linked recessive pattern.

CONCLUSIONS: This study presents a series of the oldest patients diagnosed with Progressive pseudorheumatoid dysplasia, normally a childhood disease, with an average age of 54.6. The p.Cys70Ter alteration is the most frequent pathogenic variant in Turkish patients. This study is also important in terms of showing that Progressive pseudorheumatoid dysplasia has no significant effect on life expectancy. At the same time, this study shows the progression of this disease and clinical findings that may accompany lifetime.

KEYWORDS: Arthropathy, Progressive pseudorheumatoid dysplasia, *CCN6* gene

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Kabul Tarihi / Accepted:16.06.2022
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INTRODUCTION

Skeletal dysplasia (SD) are a complex group of diseases consisting of approximately 461 different disorders that are divided into 42 groups, based on molecular and clinical findings by the latest 2019 version of the nosology. Spondyloepiphyseal dysplasia tarda, X-linked (SEDT [MIM:313400]) is characterized by disproportionate short stature, deceleration in linear growth usually beginning after the age of 5 years, and progressive joint and back pain. Multiple epiphyseal anomalies, platyspondyly, scoliosis, coxa vara, and hypoplastic odontoid process are among the accompanying radiological findings. Mutations in the localized *trafficking protein particle complex 2 (TRAPPC2* [MIM:300202]) gene in the *Xp22.2* chromosomal region are responsible for this syndrome. TRAPPC2 encodes the protein sedlin, which plays a role in vesicle transport from the endoplasmic reticulum to the golgi apparatus (1 - 4). Progressive pseudorheumatoid dysplasia (PPRD [MIM:208230]) is an autosomal recessive inherited spondyloepiphyseal disorder characterized by progressive joint pain and swelling and enlargement of joints. The disease begins around the age of 3-8 years with arthritis-like involvement of small joints of the hand. It also proceeds to kyphoscoliosis and gait disturbance by affecting shoulder and hip joints in the adolescence period (5 - 7). Mutation in the *6q21* chromosomal locus *cellular communication network factor 6 (CCN6* [MIM:603400]) gene cause this disease. This gene encodes for the CCN6 protein, a member of the connective tissue growth factor (CTGF) family, an important growth factor for normal development of the skeletal system and cartilage maturation (8 - 10).

Here we present a family with many of the typical clinical findings of these two subtypes, but also exhibited different clinical findings, in particular eye findings including myopia and astigmatism. Although all patients were male which primarily suggest X-linked spondyloepi(meta) physeal dysplasia, there was also the possibility of an autosomal recessive inheritance pattern.

MATERIALS AND METHODS

Subjects

Five patients from a consanguineous Turkish family and their four healthy relatives were included. The proband, IV.21, was a 45 years-old-male, who presented with gait disturbance, back pain and findings similar to rheumatoid arthritis in the hands **(Figure 1)**.

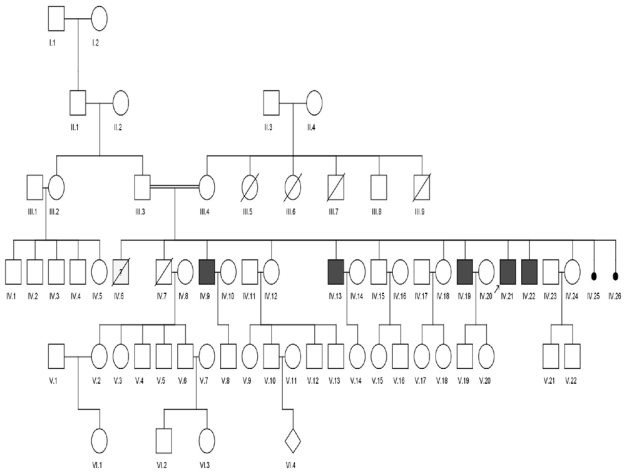

Figure1: Pedigree of the family

Patients who had a skeletal dysplasia consistent with X-linked recessive or autosomal recessive inheritance pattern as a result of history, physical examination, biochemical and radiological evaluations were included in the study.

All patients were born via normal vaginal delivery at term. Initially, growth and development were normal. The disease symptoms started at approximately 6-7 years of age, with painful nodules at the distal interphalangeal joints, and showed a severe progression, particularly in the vertebrae and hip involvement during adolescence. After the age of 20, disease progression slowed, but the patients had difficulty in walking due to inflammation in the hip joint, spine and lower extremity joints. The severe pain in the joints, including interphalangeal joints of the hands and feet, neck, back, hip and limbs, were managed with various nonsteroidal anti-inflammatory drugs (NSAIDs). The intelligence of the patients was normal. Ophthalmological evaluation of the patients revealed an astigmatism and a myopia, however one patient had a glaucoma and visual deficit of the right eye.

Cardiovascular and respiratory system examination of the patients were normal. The clinical findings of affected cases are documented in **Table 1**.

Table 1: Clinical features of all patients

Patient no	IV.9	IV.13	IV.19	IV.21	IV.22
Sex	Male	Male	Male	Male	Male
Age	65	61	51	49	47
Length (cm)	142	145	155	145	150
Weight (kg)	48	52	55	45	50
Micro / macrocephaly	No	No	No	No	No
Intelligence	Normal	Normal	Normal	Normal	Normal
Coarse face	No	No	No	No	No
Short neck	Yes	Yes	Yes	Yes	Yes
Chest deformity	Yes	No	No	No	Yes
Kyphoscoliosis	Yes	Yes	Yes	Yes	Yes
Platyspondyly	Yes	Yes	Yes	Yes	Yes
Rhizomelic shortness	Yes	No	No	No	No
Enlarged interphalangeal joints	Yes	Yes	Yes	Yes	Yes
Pain in joints	Yes	Yes	Yes	Yes	Yes
Deformation in hands	Yes	Yes	Yes	Yes	Yes
Tenderness or redness in the joint	No	No	No	No	No
Limited extension in the interphalangeal joints	Yes	Yes	Yes	Yes	Yes
Erythrocyte sedimentation rate (ESR)	Normal	Normal	Normal	Normal	Normal
Serum C-reactive protein (CRP) level	Normal	Normal	Normal	Normal	Normal
Rheumatoid factor (RF)	Negative	Negative	Negative	Negative	Negative
Hip dislocation	Not determined	Not determined	Not determined	At the age of 14-15	After trauma
Osteopenia / osteoporosis	Yes	Yes	Yes	Yes	Yes
Eyes findings	astigmatism myopia	astigmatism myopia	Glaucoma, visual deficit of right eye	astigmatism myopia	astigmatism myopia
Gait disturbance	Yes	Yes	Yes	Yes	Yes

Sample Preparation and Whole Exome Sequencing

DNA isolations were performed according to manufacturer instructions using the Anatolia Magrev Whole Blood Genomic DNA Extraction Kit, which is a magnetic bead method and the source sample was peripheral venous blood. Exome enrichment was done with the Trusight Enrichment kit (Illumina, San Diego, CA) and library preparation was done with TruSeq Rapid Exome Library Prep kit. Prepared samples were loaded onto a Hiseq 4000 sequencing system (Illumina, San Diego, CA, USA) and sequencing was done in rapid sequencing mode. Raw basecalls were demultiplexed and adapter cleaned using Illumina bcl2fastq software to obtain the clean reads in FASTQ format. FASTQ files were processed to final variant calls in accordance with the Genome Analysis Toolkit (GATK) best practices recommendations (11). In the first step, the FASTQ files (cleaned from the adapter arrays with ILLUMINA CASAVA and BCL2FASTQ) were mapped onto hg19 with BWA 0.7.15 (12). The obtained SAM file was merged with the unmapped and paired reads file with PICARD to add metadata and to convert hard clipped bases to soft clips (11). PCR and optical duplicates were marked with PICARD and later coordinate sorted with PICARD. Base quality scores were recalibrated using GATK3.8 to obtain the final BAM file ready for variant calls. Variants were called using GATK3.8 HaplotypeCaller with standard parameters and GVCF mode. Thus the call confidence of the whole reference regions were also determined (11). The resulting GVCF files were subsequently genotyped with the GATK 3.8 GenotypeGVCFs command to obtain variants. The GATK3.8 FilterVariants command was used to filter SNP and INDELs according to MQ, QD and MQRankSum values. The unfiltered variants were compared against the variant database at the XXXX Genetic Diseases Diagnostic Center to identify only rare variants in the target specimens. Rare variants were identified and then annotated with ANNOVAR and analyzed (13). The raw data obtained from the exome sequencing was annotated with various bioinformatics software such as BWA, GATK and ANNOVAR and then was exported to excel separately for each patient.

Sanger Sequencing

DNA was extracted from lymphocytes obtained from 200 µl peripheral venous blood using QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA). PCR primers for exon 2 of the WISP3 gene were designed. The forward and reverse primer sequences used were:

WISP3_2F:TTGGAGGCTGAGTGAAGAATAGTTTAAGG,
WISP3_2R:CAGTGCAATGAAAATAATGGGCTTCTG (Sequencing (5'->3')).

PCR was done for each sample in accordance with the appropriate protocol. The PCR was checked using 2% agarose gel electrophoresis (WISP3 gene 692bp) to determine whether the product could be obtained or not. Before the sequencing, the PCR products were purified using NucleoFast 96 PCR kit (Macherey-Nagel GmbH& Co. KG Düren, Germany). After completion of the thermal cycle steps, the sequence reactions were purified according to the protocol of the ZR-96 DNA Sequencing Clean-up Kit (Zymo Research Corp. USA). Capillary electrophoresis of the purified sequence products was performed using ABI 3130 (Applied Biosystems Inc.). Two gene exons and exon-intron boundaries were analyzed using SeqScape 2.5.0 (Applied Biosystems Inc.) software and sequence variations were determined.

Ethical Committee

This study was conducted in Manisa Celal Bayar University Medical Faculty Health Sciences Ethics Committee dated 28.02.2017 and numbered 1043. The study was evaluated as a research file and it was decided that it was scientifically and ethically appropriate.

RESULTS

WES was applied to the four affected cases and Sanger Sequencing was performed in one affected case and four healthy individuals. When the changes seen in more than one percent of the individuals in society were filtered out, it was seen that number of variants dropped to approximately 16000. Although pedigree analysis suggested an X-linked inheritance pattern, when the parents of the patients are consanguineous homozygous variations should be filtered according to the autosomal recessive inheritance pattern and clinical findings are taken into consideration. In order to determine the significance of these changes, firstly, variants causing premature stop codon, insertion and deletion leading to frameshift, and non-synonymous variants were filtered and annotation was performed according to the function of change. When annotating for function pathological variants were selected according to the scores of *in silico* variant analysis programs, such as SIFT Human Coding SNPs (http://sift.jcvi.org/www/SIFT_chr_coords_submit.html), Mutation Taster (http://www.mutationtaster.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/dbsearch.shtml). All changes detected after these stages were visualized separately in excel format for each patient. VarSome (https://varsome.com/) combined database, the human genomic variant search engine, was used to detect the pathologic variation that caused the disease. Variations dropping below 10 in the filtration process were subjected to screening using the VarSome database.

All these annotation procedures resulted in two changes that were in entire agreement with the patient's clinical findings and inheritance pattern. It was observed that the c.210C>A; (p.Cys-70Ter) **(Figure 2, 3)** nonsense homozygous variant and c.302G>A; (p.Gly101Glu) **(Figure 4, 5)** missense homozygous variant in the exon 2 of the NM_198239.1 transcript of the *CCN6* gene were pathological mutations causing the disease (rs121908901, rs147337485).

Approximately 125,000 variants obtained from whole exome analysis were filtered, finally identifying two variants, and leading to a diagnosis of autosomal recessive inherited PPRD. Sanger sequencing analysis of all control cases showed that the mother and the other brother were carriers, while the other two healthy relatives did not carry both mutations.

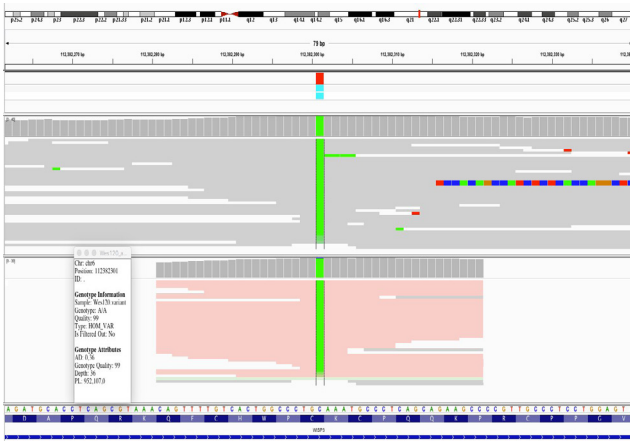

Figure 2: Exome sequencing image of the all patients revealed a c.210C>A homozygous mutation in the *CCN6* gene

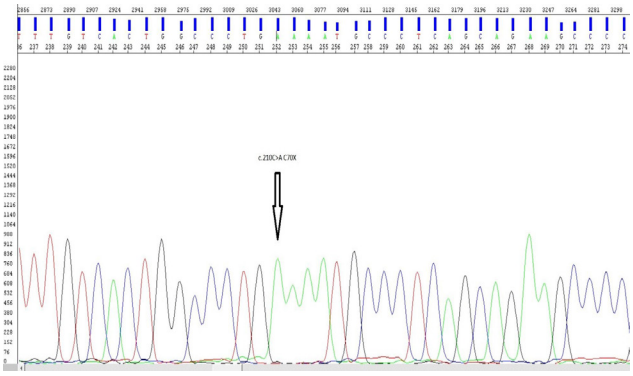

Figure 3: Sanger sequencing confirmation of the all patients revealed a c.210C>A homozygous mutation in the *CCN6* gene

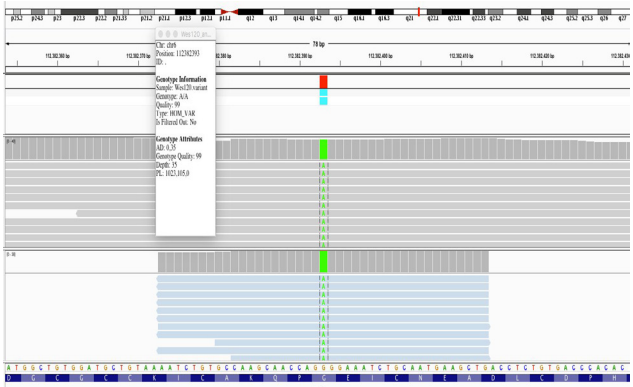

Figure 4: Exome sequencing image of the all patients revealed a c.302G>A homozygous mutation, in the *CCN6* gene

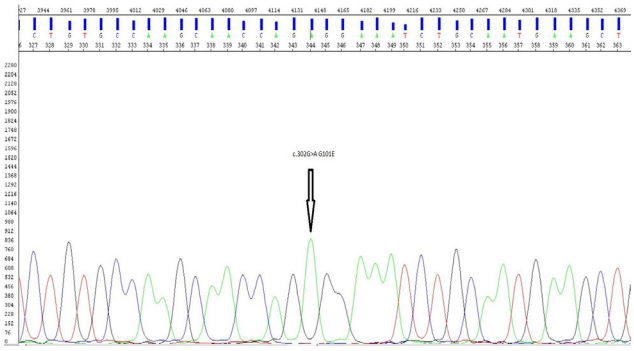

Figure 5: Sanger sequencing confirmation of the all patients revealed a c.302G>A homozygous mutation in the *CCN6* gene

DISCUSSION

This study was performed to determine the genetic etiology of patients with undiagnosed skeletal dysplasia for years. After whole exome sequencing analysis two homozygous mutations in the second exon of the CCN6 gene confirmed the diagnosis of PPRD, according to the National Center for Biotechnology Information (NCBI) reference sequence, NM_198239.1. One of these mutations, c.210C>A, is predicted to cause a premature stop codon, p.Cys70Ter. This mutation was previously reported in the Human Genome Mutation Database-Public Version (HGMD public) with the code CM991252. The c.210C>A mutation has been reported from several countries, including Italy, France, Lebanon, Syria, Turkey, Germany, India and Iran (6, 9, 14-19). This mutation accounts for almost 28% of the pathogenic variants associated with PPRD. It is the most frequent mutation in the Turkish population (20). Surprisingly, the c.302G>A; (p.Gly101Glu) missense amino acid mutation was detected in all patients, and was present in the same coding region as the other mutation. It is present in Human Genome Mutation Database-Public Version (HGMD public; code CM129541) and has been formerly reported (6, 16, 18, 21). This mutation results in substitution of a Glycine, present in the wild type protein, with a Glutamine at position 101. The glycine, the most flexible of all residues, has markedly different physicochemical properties from glutamine so that the presence of glutamine in the mutant can disturb the normal function of this domain and potentially abolish its function (22). The wild-type and mutant amino acids differ in size and the mutant residue is bigger, which may lead to changes in tertiary and quaternary structure of the mutant protein (22). The torsion angles for this residue are unusual, and only glycine is flexible enough to maintain these torsion angles. Thus, mutation with another residue will force the local backbone into an incorrect conformation and will disturb the local structure.

One of the functions of *CCN6* protein is to bind to Insulin-Like Growth Factors. Insulin-like growth factors are involved in cell growth regulation and are associated with high affinity, thrombospondin type 1 repeat domains (TSP1) and a C-terminal cysteine node-like domain (CT). Both mutations detected were located within an IGFBP N-terminal domain, annotated in UniProt. The association of p.Cys70Ter and p.Gly101Glu mutations was previously reported by Delague et al. (18), Rai et al. (6) and Temiz et al. (16) and it was postulated that this might represent a founder effect (6, 16, 18). Consanguineous marriages may cause the emergence of these homozygous mutations. Most PPRD patients with p.Cys70Ter and p.Gly101Glu homozygous mutations have exhibited similar phenotypes. In the current pedigree, the ocular findings including astigmatism and myopia, have not previously been reported in PPRD. Members of this family had a clinical picture resembling both syndromes. Their pedigree had two possible inheritance patterns, X-linked recessive and autosomal recessive inheritance, as all females were healthy and all males were affected. As mental capacity was invariably normal, we hypothesized that a syndrome with X-linked recessive inheritance pattern was the most likely diagnosis initially but, it was decided to perform exome sequencing because an exact differential diagnosis was not possible.

This study has highlighted the importance of whole exome analysis to diagnose heterogeneous and multifactorial diseases, such as skeletal dysplasia. This study is also present the oldest group of patients to date, diagnosed with PPRD, which is usually a disease diagnosed in childhood and is thus important as PPRD in this pedigree had no effect on life expectancy.

ACKNOWLEDGEMENT

All authors contributed equally to this study. All individuals participating in the study were informed about the study and an informed consent

form was obtained. We thank all the individuals who took part in this study. I would like to thank my esteemed teachers Serdar Ceylaner, Assoc. Prof., MD. and Gülay Ceylaner, Assoc. Prof., Ph.D. who supported me in this study and the entire team of INTERGEN Genetics and Rare Diseases Diagnosis Research & Application Center.

This study was supported financially by the Scientific Research Projects Coordination Unit of Manisa Celal Bayar University (project no. 2017-075).

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