**Exome sequencing for the differential diagnosis of ciliary chondrodysplasias**

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**Abstract:**

*Exome sequencing is becoming widely popular and affordable, making it one of the most desirable methods for the identification of rare genetic variants for clinical diagnosis. Here, we report the**clinical application of whole exome sequencing for the ultimate diagnosis of a ciliary chondrodysplasia case presented with an initial clinical diagnosis of Asphyxiating Thoracic Dystrophy (ATD, Jeune Syndrome). We have identified a novel homozygous missense mutation in WDR35 (*c.206G>A)*, a gene previously associated with Sensenbrenner Syndrome,* Ellis-van Creveld syndrome *and Short- rib polydactyly syndrome type V. The genetic findings in this family led to the re-evaluation of the initial diagnosis and a differential diagnosis of Sensenbrenner Syndrome was made after cautious re-examination of the patient. Cell culture studies revealed normal subcellular localization of the mutant WDR35 protein in comparison to wildtype protein, pointing towards impaired protein-protein interaction and/or altered cell signaling pathways as a consequence of the mutated allele. This research study highlights the importance of including pathogenic variant identification in the diagnosis pipeline of ciliary chondrodysplasias, especially for clinically not fully defined phenotypes.*

**Keywords:** Ciliary chondrodysplasias; Exome sequencing.

1. **Introduction**

The rise of next generation sequencing (NGS) technologies over the past few years has transformed the (clinical) genetics setup. NGS gene panels, whole exome and whole genome sequencing have been successfully used for unraveling the molecular mechanisms underlying hereditary diseases. Identifying the causative pathogenic genetic variant now serves as a diagnostic tool for many diseases [1].

“Ciliopathies” are highly heterogeneous multi - organ disorders caused by functional and structural abnormalities in cilia [2]. ‘Ciliary chondrodysplasias’ are ciliopathies affecting skeletal development in mammals as a consequence of a defective primary cilium and/or impaired cell signaling pathways regulated by ciliary proteins[3]. Short- rib polydactyly syndrome (SRPS; MIM IDs.611263, 613091, 263520, 269860, 614091), Jeune Asphyxiating thoracic dystrophy or Jeune syndrome (JATD MIM 208500), Ellis-van Creveld syndrome (EVC; MIM 225500), Mainzer-Saldino syndrome (MZSDS; MIM 266920), Sensenbrenner syndrome or Cranioectodermal dysplasia (CED; MIM 218330), Oral facial-digital syndrome 4 (OFD4; MIM 258860) and Weyers acrodental dysostosis (WAD; MIM 193530) are ciliary chondrodysplasias with overlapping clinical features. Except for WAD, all these ciliary chondrodysplasias show an autosomal recessive inheritance pattern. They are characterized by skeletal defects such as short ribs, narrow thorax and brachydactyly, often combined with craniofacial abnormalities, ectodermal dysplasia, polydactyly as well as variable liver, kidney, brain and retinal abnormalities. SRP subtypes are more severe in this subgroup resulting usually in neonatal death[3]. A summary of the major clinical features of ciliary chondrodysplasias and the underlying genetic defects are shown in table 1.

As no protein synthesis occurs within the cilium, intraflagellar transport (IFT) is vital to build and maintain the ciliary structure. IFT is an ATP dependent transport process governed by large multiprotein complexes, IFT complex A and B. *WDR35* (WD Repeat Domain 35) encodes an intraflagellar transport (IFT) protein within complex A and pathogenic *WDR35* variants have been previously associated with CED[4] SRPS[5] & EVC[6] syndromes.

Sensenbrenner syndrome or Cranioectodermal dysplasia (CED) is a rare multi-organ disorder with dolicocephalus skull resulting from the craniosynostosis of the sutura sagittalis, scaphocephaly, skeletal deformities (narrow thorax, brachydactyly and terminal hypoplasia of the fingers) and ectodermal abnormalities (sparse hair, hyper pigmented hair, Hypodontia/microdontia and finger/toe nail dysplasia). Additional features include joint laxity, growth retardation, single transverse palmar crease and characteristic facial features with frontal bossing and low set ears. The visceral anomalies include nephronophthisis, hepatic fibrosis and retinal dystrophy [7-9]. Understanding of CED phenotype is limited with less than 60 individuals reported to date. Biallelic mutation in *IFT122 [10]*, *WDR35 [11]* , *WDR19 [12]* or *IFT43 [13]* , have been reported before in CED cases.

The major clinical features of JATD are narrow chest (less severe compared to SRPS but usually more severe then what is observed in CED), brachydactyly, short limbs and variable visceral anomalies such as nephronophthisis-like kidney disease, retinal degeneration and rarely liver disease [3].

Making a definitive clinical diagnosis can be difficult within the spectrum of ciliary chondrodysplasias, especially at the fetal and/or neonatal stage, when ectodermal defects such as hair and teeth abnormalities and visceral symptoms may not be evident yet. Here, we report a study where the identification of the underlying genetic defect by exome sequencing helped us to revise the initial clinical diagnosis made during infancy.

In this study, we describe a homozygous missense mutation in *WDR35* in a Kuwaiti child with the clinical diagnosis of Jeune Asphyxiating thoracic dystrophy (JATD). The genetic diagnosis led to the revision of the initial diagnosis to CED, underpinning the power of genomic sequencing technologies for genetic medicine. Further, in this report, we also present an overview of causative WDR35 mutations and associated phenotype features to date.

1. **Materials and methods**
   1. *Patient*

Informed consent and minor assent (participants below 21 year) was obtained from the family according to the regulation of Dasman Diabetes Institute (DDI) ethics committee.

* 1. *Exome sequencing and reassessment of the affected case.*

For exome sequencing, sheared high quality genomic DNA of the index case [Covaris, Woburn, MA, USA] was subjected to Illumina Paired End DNA Library preparation using Truseq DNA sample prep kit following manufacturer’s recommendation (Illumina Inc., USA). The DNA libraries were then enriched for exomes using Illumina Truseq Exome Enrichment kit, which covers 97.2% of CCDS coding exons (Illumina Inc., USA) following manufacturer’s instructions. Quality of libraries throughout the protocol was assessed using Agilent 2100 High sensitive DNA chips (Agilent Technologies, Inc., USA). Enriched libraries were then subjected to standard Illumina protocols for cluster generation using cBot (Illumina Inc., USA) on a version3 Illumina Paired End Flow cell (Illumina Inc., USA). The clustered libraries were then sequenced using Hiseq 2000 platform (Illumina Inc., USA) as paired end 100base pair reads.

Reads were aligned to the human reference genome, hg19 using BWA v.0.5.9r16[14]. Realignment around known indels from the 1000 Genomes Pilot study and recalibration of base quality scores was performed with GATK 1.1.5 [15]. Variant calling was done using SAM tools mpileup v.0.1.17 [15] and Unified Genotyper [16] .

The variant call files of the affected individual along with in-house controls were then uploaded in Ingenuity Variant Analysis software (QIAGEN; Redwood, CA, USA) and analysed following the default filter cascade (Suppl. table 1& Suppl. table 2). The filters were adjusted to identify the variants which are rare, deleterious, inherited in autosomal recessive pattern and related to ciliary function. Since some of the ciliary protein genes are missing from the ingenuity database ciliopathy gene list, we uploaded the ciliary chondrodysplasia gene list also for analysis (Suppl. table 3.). The quality of the sequence reads was assessed using Integrative Genomics Viewer Software (Broad Institute, MA, United States).

# Targeted Sanger sequencing was performed to validate the exome sequencing results. Briefly, the region of interest was amplified using ‘Go Taq Green Master Mix’ (Promega). The amplified fragments were then sequenced in an ABI Prism 3730xl Genetic Analyser using the standard procedures. The sequences were then analysed using the Chromas Pro Software (Technelysium Pty Ltd).

# Single nucleotide polymorphisms (SNP) searches were performed in the National Council for Biotechnology Information dbSNP database (http.//www.ncbi.nlm.nih.gov/SNP), the 1000 Genomes project (http.//browser.1000genomes.org), the Exome Sequence Project’s Exome Variant Server ([http.//evs.gs.washington.edu/EVS)](http://evs.gs.washington.edu/EVS)) and Exome Aggregation Consortium (ExAC), Cambridge, MA (http.//exac.broadinstitute.org) (December 2015).

* 1. *Cell culture Experiments.*

Site Directed Mutagenesis, DNA sub-cloning into mammalian expression vectors containing eCFP and mRFP fluorescent tags and hTERT-RPE and HEK293T cell culture including induction of ciliation due to serum starvation and subsequent microscopy imaging was performed under standard conditions as previously described [17-20]. Primer and plasmid sequences are available on request.

1. **Results**

Our index patient was a 9-year-old female child born to healthy, consanguineous Kuwaiti parents, who initially presented with a diagnosis of Jeune Asphyxiating thoracic dystrophy. The affected case had a liver and kidney transplantation four years back and as of today, the girl is stable except with occasional episodes of respiratory infections and an enlarged thymus.

The exomes were sequenced at a mean coverage of 30X. Exome data is deposited in NCBI Sequence Read archive (SRP068602). After data analysis using Ingenuity Variant Analysis software, a novel homozygous variant in *WDR35* at position c.206G>A (p.G69D) was identified in the (proband IV. 2; Figure 1) as pathogenic and the probable cause of disease starting from 331517 variants in total. No other pathogenic variants

were identified from the candidate gene list of ciliary chondrodysplasia genes (Suppl. table 3).

Sanger Sequencing confirmed the homozygous change in *WDR35* in the index patient and the affected sibling, further segregation analysis also confirmed the autosomal recessive inheritance pattern. Both parents were heterozygous for the change and an unaffected sibling was homozygous for the wild type allele (Figure. 1). The variant was also not detected in 94 Kuwaiti healthy controls (188 alleles) excluding the possibility of being an ethnic-specific rare polymorphism.

The variant was predicted to be damaging by both SIFT and PolyPhen, two publically available software that predict loss- or gain-of-function mutations. The homozygous change in *WDR35* at position c.206G>A (p.G69D) is reported in dbSNP database (rs765513105) with no associated minor allele frequencies and is not reported in the ExAc database which comprises of 60,000 exomes or the NHLBI Exome Sequencing Project (ESP) with ~13,000 exomes. However, recently the same variant was reported as causative mutation in 2 affected girls from Arabian decent with a similar although not identical clinical phenotype including acromelia, epiphyseal changes, polycystic kidney, liver disease and cerebellar hypoplasia [21]. We don’t know of any relation of our Kuwaiti family with the Arabic family reported by Shaheen et al., however it is possible that both families have a common ancestor or that the allele is a rare middle Eastern founder allele.The change lies in a WD40 repeat domain (5). WD40 domains are protein-protein interaction domains, in the case of *WDR35* those domains are likely often involved in intracellular trafficking, cargo recognition and binding.

Pathogenic variants in *WDR35* have been previously reported for CED (most frequently) as well as a family with SRPS and recently in some EVC cases but not JATD. A summary of reported variants in *WDR35* (Figure 2), along with their clinical features are shown in table 2. Detection of the *WDR35* variant therefore prompted us to clinically re-examine the index case and compare the clinical data with that of the overlapping ciliary chondrodysplasias. We further performed a comprehensive radiological study on the skeletal features of the 9-year-old patient. This revealed a dolichocephalic skull shape with frontal bossing and premature sagittal suture fusion with coronal and lamboid sutures showing indistinct margins, suggestive of partial fusion while the sella, calvarial bone and base of the skull appeared normal. Metacarpal and phalangeal bones of the hand was short and stubby. The proximal phalangeal epiphyses showed evidence of tunneling into metaphyses. The cortices were normal and no evidence of periosteal reaction was seen. The thoracic cage appeared normal without evident abnormalities of the ribs, clavicles or pelvis. However, physical examination of the patient revealed ectodermal anomalies like teeth and nail dysplasia (Figure 3).

In order to further investigate the biological consequences of the identified *WDR35* mutation, we proceeded to recapitulate the mutation using site directed mutagenesis and express wildtype and mutant *WDR35* in both renal tubule (HEK293T) as well as retinal pigment epithelium (RPE) cells (Figure 4). Both proteins mainly localized to the base of primary cilia with occasional additional axonemal presence, indicating that the observed patient phenotype does not result from mislocalised mutant protein but more likely from impaired protein-protein interactions e.g. with other IFT proteins and/or IFT cargo or from effects on cellular signaling pathways such as hedgehog signaling.

1. **Discussion.**

We performed whole exome sequencing (WES) in a case with the clinical diagnosis of JATD made in infancy and to our surprise, the genetic results revealed a homozygous missense allele in *WDR35*, a gene not previously associated with JATD but in contrast with CED, EVC and SRPS. The radiological study performed as part of the clinical re-examination after sequencing revealed dolichocephalic skull with frontal bossing, which is a hallmark of Sensenbrenner syndrome. The thorax of the affected case looked normal in contrast to what is usually observed in JATD patients, however the thorax appearance becomes more normal with increasing age also in JATD. The affected case had pulmonary valve stenosis with left ventricular hypertrophy, in concordance with EVC cases. Ectodermal defects as observed in our patient are found in CED as well as EVC but not JATD. Liver and renal diseases are not observed in EVC cases [22], but the affected individual in this family had end stage liver and kidney diseases. The pelvis appeared normally configured, however pelvis abnormalities usually observed in SRPS, JATD and EVC such as trident acetabulum with spurs is often only evident within the first year of life. The ectodermal dysplasia, visceral anomalies and the cranial features aided us to make the definitive diagnosis of Sensenbrenner Syndrome correcting the initial diagnosis.

*WDR35* is one of the most common gene associated with CED. All reported variants of *WDR35* to date and the associated clinical phenotype, along with the index case from this study are illustrated in figure 3 and table 2. Interestingly, our cases shares many but not all clinical features with the Arab case reported by Shaheen et al [21] carrying the same homozygous mutation. A correlation with molecular and clinical phenotype is difficult to derive in these ciliary chondrodysplasias.

In the era of personalized medicine, quick and timely diagnosis of genetic disease is more important than ever as the precise diagnosis governs the clinical management. Prenatal diagnosis based on 3D ultrasound is widely used to identify developmental anomalies. Taking into account nuchal translucency (NT) thickness, endocardial cushion defect, narrow or bell shaped thorax, polydactyly, micrognathia, frontal bossing skull and other skeletal features sonogram aids to diagnose skeletal dysplasias [23]. However the prenatal identification is not infallible, and many of the phenotypes evolve during the later pregnancy. Further, extensive phenotypic overlap between different chondrodysplasias such as EVC, JATD, SRPS and CED regarding features visible on prenatal ultrasound (e.g. shortened limbs, narrow thorax) often makes a precise diagnosis prenatally impossible without the help of genetics. However, such precise diagnosis is very helpful for the clinical management of affected families: for example, the likelihood of experiencing lethal or life threatening respiratory problems is a lot higher in SRPS and JATD compared to MZSDS and CED. In contrast, MZSDS and CED patients are far more likely to develop kidney and liver disease during infancy. In this context, advent of Next Generation Sequencing is not only revolutionizing the biomedical research but also clinical practice [3; 17; 24; 25; 26] .

1. Conclusion:

# In this study, the identification of pathogenic genetic variant, made us to correct the initial clinical diagnosis. NGS techniques are becoming affordable and efficient, making it accessible to all research and diagnostic facilities. In line with the current study, the identification of causative gene also should be included as a clinical prognosis strategy for better patient care.

**Competing interests**

The authors declare that there are no conflict of interest.

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