



EXOME SEQUENCING REVEALS CAUSATIVE VARIANTS IN FOUR GENES ASSOCIATED WITH NEURODEVELOPMENTAL DELAY

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Abstract

Neurodevelopmental disorders (NDDs) represent a diverse category of diseases that are associated with impaired brain development and functioning, resulting in intellectual disability (ID), developmental delay (DD), autism spectrum disorders (ASDs), epilepsy, and diverse other neurological impairments. Consanguinity offers a useful background to detecting the presence of autosomal recessive (AR) mutations that cause these conditions using whole-exome sequencing (WES). This study was designed to find the genetic variants of NDDs in consanguineous families. Four consanguineous Pakistani families with various NDD phenotypes were sequenced using whole-exome sequencing. Causative mutations were identified using variant filtering and segregation analysis, and in silico prediction of pathogenicity. A total of four pathogenic variants were found: a homozygous nonsense mutation (c.3978G>A; p.Trp1326* *ASPM* in PKND3) leading to a premature stop codon at Trp1326; a nonsense mutation (c.57G>A, p.Trp19* *SRD5A3* in PKND5, an upstream splice -site mutation (c.-48+1G>C, p.? *SNX14* in PKND6), a hemizygous in-frame deletion c.1222-1224del p.Phe408del in the *SLC6A8* gene was identified in the PKND8 family. This study expands the range of mutations associated with known NDD-related genes by proposing *SLC6A8* as a possible novel pathogenic variant. The results support the diagnostic value of WES in consanguineous families and contribute to the molecular understanding of autosomal recessive neurodevelopmental defects.

Keywords: Neurodevelopmental disorders; Whole-exome sequencing; Autosomal recessive; *ASPM*; *SRD5A3*; *SNX14*; *SLC6A8*

Introduction

The Neurodevelopmental Disorders (NDDs) represent an extensive spectrum of disorders that prevent the neurodevelopment of the nervous system, leading to the impairment of cognition, behavior, motor skills, and adaptive functioning (Srivastava et al., 2019). The clinical spectrum comprises intellectual disability, autism spectrum disorders, attention-deficit hyperactivity disorder (ADHD), and a myriad of syndromic entities that impair neurological functioning. The prevalence rates worldwide are estimated between 1 to 3 percent, but there were differences in different regions because of ethnic, sociocultural, economic, and consanguineous marriage. Environmental and genetic factors have a role in the NDDs, and the most prevalent etiology is pathogenic genetic variation. Lots of these variants are responsible for NDDs caused by consanguinity and display as

autosomal recessive phenotypes. Consanguineous ratios are more than 60 percent in Pakistan (Srivastava et al., 2019).

The NDDs are genetically heterogeneous, with over 1,000 genes listed in the Online Mendelian Inheritance in Man. *ASPM* autosomal recessive mutations are a significant cause of primary microcephaly and are typically 40-50% of reported NDD cases (Létard et al., 2018). *SRD5A3* mutations cause a specific form of glycosylation congenital disorder, with intellectual disability and ocular defects (Cantagrel et al., 2010), whereas *SNX14* mutations define a syndrome of cerebellar atrophy with coarse features (Akizu et al., 2015; Thomas et al., 2014). X-linked creatine transporter deficiency is a second most common X-linked cause of intellectual disability after Fragile X syndrome caused by variants in *SLC6A8* (Wang et al., 2018).

Although there are advances in the discovery of genes, a lot of NDDs genes remain undiagnosed, yet to be discovered. Whole-exome sequencing was used to study four consanguineous Pakistani families with various NDD phenotypes in this study. Three of the above-mentioned pathogenic variants were as previously reported, while *SLC6A8* was suggested as a novel pathogenic variant in neurodevelopmental disorders.

Materials and Methods

Study Participants and Ethical Approval

F Pakistani consanguineous families (PKND3, PKND5, PKND6, and PKND8) were recruited from the remote areas of Khyber Pakhtunkhwa, Pakistan. Within each family, several affected individuals had symptoms of a neurodevelopmental disorder. Participants were only recruited to take part in research, and informed consent in writing was received by all the participants or their legal guardians. Pedigrees were built in accordance with the conventional approach (Bennet, 1995). The research was done in the protocol granted by the Department of Biological Sciences, Faculty of Sciences, International Islamic University Islamabad, as per the Declaration of Helsinki.

2.2 Clinical Examination and Blood Samples.

Preclinical assessment was done using a standardized proforma. To remove the confounding effect of the environment, maternal and perinatal histories were collected among the guardians of the respective affected probands. The informal consent was taken, and blood samples (5 mL in adults and 2 mL in children) were obtained in EDTA tubes.

Table 1. Overview of the preclinical manifestation of recruited families

Clinical Features	PKND3	PKND5	PKND6	PKND8
Consanguinity	+	+	+	+
Autism Spectrum Disorder	-	-	-	+
ADHD	-	-	-	-
Developmental delay	+	+	+	+
Language delay	+	+	+	+
Motor delay	+	+	+	+
Intellectual disability	+	+	+	+
Microcephaly	+	-	-	-
cerebellar dysfunction	N/A	N/A	N/A	N/A
Hypotonia	-	-	-	+
Behavioral problems	+	-	-	+
Ataxia	N/A	N/A	N/A	N/A

Clinical Features	PKND3	PKND5	PKND6	PKND8
Dystonia (Movement)	-	-	+	-
dysmorphic features	+	+	+	-
GIT issues	N/A	N/A	N/A	N/A
Brain MRI Availability	-	-	-	-

Abbreviations: +: present; -: absent; N/A: not available; ADHD: Attention Deficit Hyperactivity Disorder; GIT: Gastro intestinal

2.3 Whole Exome Sequencing and Bioinformatic Analysis.

A Twist Human Core Exome kit was used to perform whole-exome sequencing on individuals. BWA -MEM (v0.7.17) was used to read the data, and then it was duplicated using Picard (v2.15.0) and indel realignment/base quality recalibration (GATK v3.7.0). GADK Haplotype Caller was used to do variant calling, and Alamut Batch v1.8 or v1.10 was used to do annotation. GATK Depth of Coverage was used to measure read depth. Both Exome Depth and SavvyCNV were used to identify copy-number variants and followed the Best Practices of GATK. Homozygous variants that had minor allele frequencies less than 0.01 were prioritized in variant filtering in publicly available databases, including gnomAD, ExAC, and the 1000 Genomes Project. ANNOTO was used to make annotations, and classification was based on the ACMG guidelines (Richards et al., 2015). The analysis of segregation was carried out in the available members of the family to ensure the pattern of inheritance and the cosegregation with the disease phenotype.

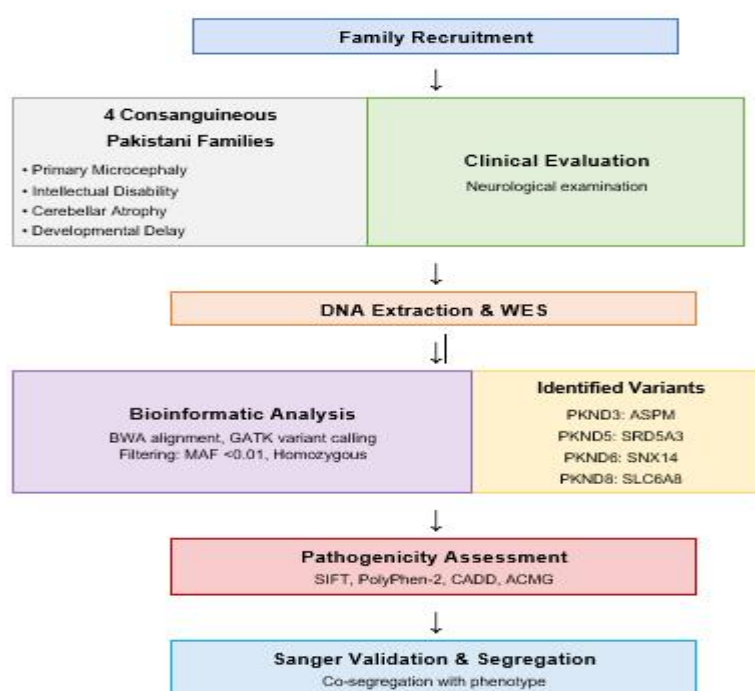


Figure 1. Study Workflow and Variant Identification Pipeline

Results

Four consanguineous families having ND were recruited and analyzed. WES was performed on one proband chosen randomly from each family. Three previously published known mutations, one novel mutation in a previously reported gene, and one novel candidate gene mutation were revealed. Co-segregation was performed to validate the findings through Sanger sequencing.

PKND3

A four-generation family with two generations of consanguinity, having two affected monozygotic male III:2, and IV:2, were enrolled with other family members available.

Both affected individuals suffered congenital microcephaly with significantly diminished head circumference and a sloping forehead. Clinical severity was variable, with one having mild intellectual disability and the other severe intellectual handicap with developmental delay, speech and hearing, seizures, and aggressive behaviors. Sanger sequencing segregation of *ASPM* confirmed a homozygous missense mutation, c.3978G>A (p.Trp1326), in both cases. The mutation is an exonic nonsense mutation, as it is in exon 15 and contains a premature stop codon that truncates the *ASPM* protein and interferes with normal neurogenesis. The variant was present in a heterozygous form in all the unaffected family members. This particular mutation has been observed over and over again in the Pakistani and Indian families and indicates that there is a regional founder effect and that exon 15 of *ASPM* is a mutational hotspot in primary microcephaly.

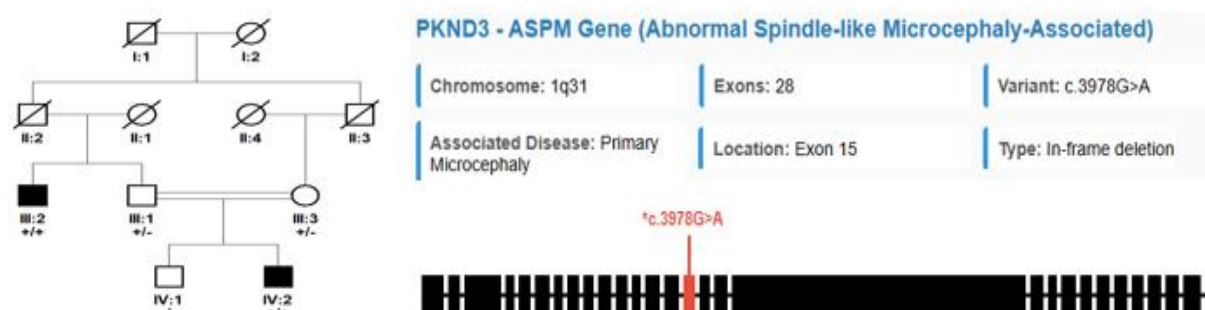


Figure 2. PKND-3 pedigree. Empty Square shows male; dark filled shows affected, double line shows consanguinity, and cross line shows deceased; -/- = wild type; +/- = heterozygous carrier; +/- = mutant

Family PKND5

A consanguineous two-nuclear family in the same family was enrolled. Three offspring were affected, showing the same phenotypic features. Consent forms were signed to participate in this study.

Proband IV:2 and IV:4 shows intellectual disability, communication disorder, nystagmus, and visual impairment. Proband IV:4 also shows difficulty in walking.

Genetic Findings

Proband IV:4 were selected for whole exome sequencing to find the genetic variants. A total 44 homozygous regions were identified. These variants were further screened out according to the proband phenotype. Nonsense mutation in *SRD5A3* gene Chr4(GRCh37):g.56212560G>A, NM_024592.5:c.57G>A, p.Trp19* were identical to the phenotypic features of the proband IV:4. *SRD5A3* gene encodes a protein that plays a key role in the production of androgen 5-alpha-dihydrotestosterone (DHT) and activation pathway maintenance of the androgen-androgen receptor. This protein also converts the polyprenol into dolichol, dolichol then links oligosaccharides and monosaccharides, a precursor used for N-linked glycosylation of proteins. Alteration occurs in the amino acid Tryptophan TGG pattern, the third nucleotide is replaced by A, which causes to stop further translation, and thus the immature/incomplete protein formed leads to congenital disorder of glycosylation type Iq. These mutations are previously reported for the congenital disorder of glycosylation and kazhiri syndrome.

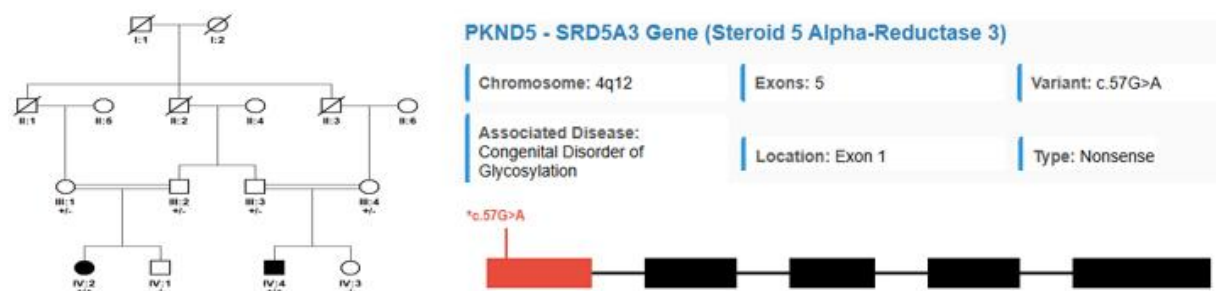


Figure 3. PKND5 pedigree. Red label on chromosome pinpoint the mutation; -/- = wild type; +/- = heterozygous carrier; ++ = mutant

PKND6

A consanguineous family with three siblings affected in this family. Two sisters and one brother. Both sisters show more severe phenotypic symptoms than their brothers. All of them had periodic epilepsy, and the sisters IV:2 and IV:4 had seizures five to six times a week. The proband IV:4 was macrocephalic with abnormal facial.

Proband IV:2 was selected for exome sequencing. A total 31 homozygous genes were identified through exome sequencing. By filtering out the *SNX14* gene variant Chr6 (GRCh37):g.86256801C>G splice upstream c.-48+1G>C, p.?. The identified gene variants were checked through different in-silico predicting tools for the pathogenicity score. OMIM database shows the gene is reported for the same disorders before in many families.

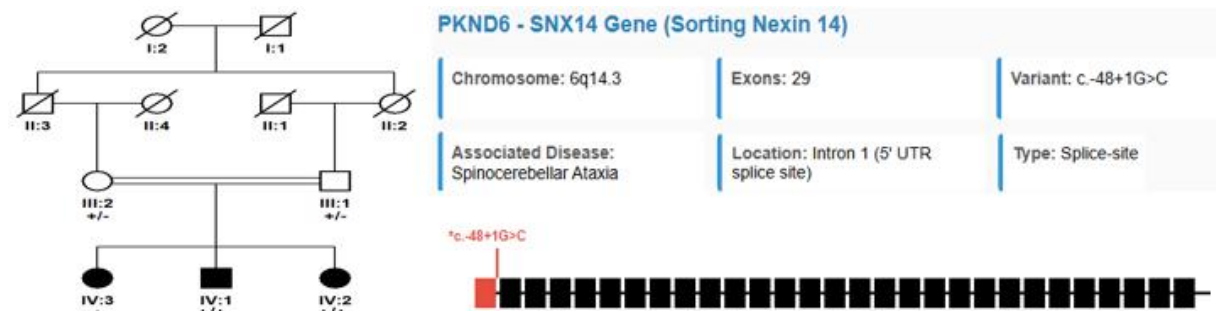


Figure 4. PKND6 pedigree. Red arrow on chromosome = Location of mutation in *SNX14*, intron 1

PKND8

This four-generation family belongs to Buner, Khyber Pakhtunkhwa. The family was ascertained and enrolled at their residence. Family details were taken from the parents. Four-generation family pedigree was drawn. Blood samples were taken from two affected (IV:1, IV:2), and their parents (III:1, III:2).

4.8.2 Clinical Features

Proband IV:1 and IV:2 were 8 and 5 years old at the time of the visit. Both probands show developmental delay, hearing impairment, speech delay (unable to talk), Aggressive behavior, showing deterioration in social behavior through his dress.

Proband IV:5 was selected for whole-exome sequencing to find the genetic variant/s. Thirty-six homozygous regions were found in exome sequencing. By filtering out the variants, out of thirty-six, only one variant, the *SLC6A8* gene variant, matches the clinical features of proband IV:2. The *SLC6A8* gene variant shows a hemizygous X-linked recessive inheritance pattern. In frame deletion in exon 8 position ChrX (GRCh37): g.152959440_152959442del, NM_005629.4:c.1222_1224del, NM_005629.4:p.Phe408del occurred. *SLC6A8*'s normal function is to provide instructions to make the sodium and chloride-dependent creatine transporter 1 protein. This protein transport compound,

creatine into cells which is important to use and store energy properly in the body. The creatine transporter protein mediated creatine uptake. *SLC6A8* gene mapped to Xq28; it consists of 13 exons, spans ~8.4 kb, and encodes a protein of 635 amino acids. A mutation in the *SLC6A8* gene can cause creatine transporter deficiency syndrome 1, which affects the brain. Deletion of the amino acid at position p. Phe408del, which make Phenylalanine, and it stops further translation.

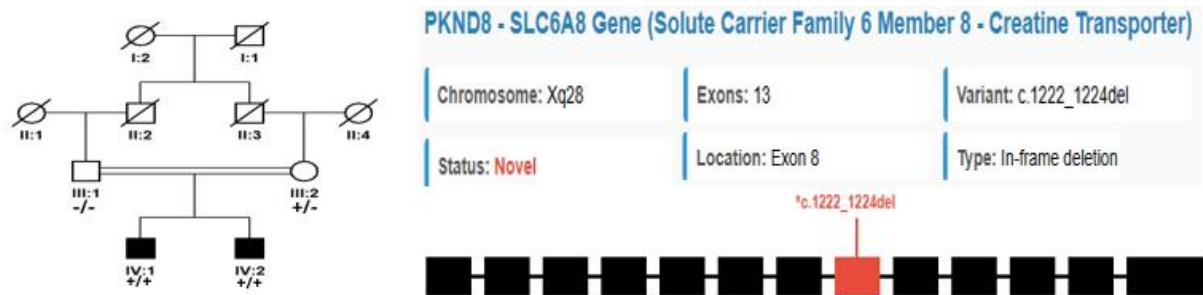


Figure 6. PKND-8 pedigree. Red color on exon 8 locate the mutation spot at *SLC6A8* Gene

A summary of identified variants and their classification is shown in **Table 2**.

Family ID	Chromosome	Gene	Protein variant	Mutation effect	Reported/Novel	Zygosity	Reference
PKND3	1:197087006C>T	<i>ASPM</i>	p.Trp1326*	Stop gain	Reported	Homo	Khan et al. (2017)
PKND5	4:56212560G>A	<i>SRD5A3</i>	p.Trp19*	Stop gain	Reported	Homo	Cantgrel et al. (2010)
PKND6	6:86256801C>G	<i>SNX14</i>	p.(?)	Splice-site	Reported	Homo	Thomas et al. (2014)
PKND8	X:152959440_152959442del	<i>SLC6A8</i>	p.Phe408del	In-frame deletion	Novel	Hemi	This study

Abbreviations: Homo= homozygous; Hemi= hemizygous

- **Notes:**
- *ASPM* variant c. 3978G>A creates a premature stop codon at position 1326 (exon 15 of 28 exons).
- *SRD5A3* variant c. 57G>A creates an early nonsense mutation at codon 19 (exon 1 of 5 exons).
- *SNX14* variant c. -48+1G>C affects the canonical donor splice site of the 5UTR intron.
- *SLC6A8* variant c. 1222_1224del is a novel mutation causing in-frame deletion of phenylalanine at position 408.

All variants were validated according to HGVS 9HUMAN genome Variation Society) nomenclature guidelines

Discussion

This study has used whole-exome sequencing (WES) to investigate the genetic basis of neurodevelopmental disorders (NDDs) among five consanguineous families from Pakistan with 100% diagnostic yield. The higher diagnostic ratio increases the efficacy of WES in populations with high consanguinity, as homozygosity mapping effectively reduces the search space for causative variants. Our findings are consistent with earlier research from Pakistan, which indicates diagnostic yields of 35 to 60% in consanguineous families with NDDs (Létard et al., 2018).

The *ASPM* variant, which was described in family PKND3, broadens the mutational spectrum of this gene. Mutations in *ASPM* are the leading cause of autosomal recessive primary microcephaly,

representing 40% to 50% of MCPH cases worldwide (Abdel-Hamid et al., 2016; Létard et al., 2018). The gene encodes the Abnormal Spindle-like Microcephaly-associated protein, which localizes to the centrosome of apical neuro-progenitor cells and is essential for positioning of the spindle pole during neurogenesis. These findings are in line with the laid-down phenotype of severe microcephaly, intellectual disability, and simplified gyral pattern reported in previous studies (Nicholas et al., 2010).

The *SRD5A3* nonsense variant p.Trp19* detected in the patient in family PKND5 has been described in several unrelated families with *SRD5A3*-CDG previously (Cantagrel et al., 2010; Kara, Ayhan, Gökçay, Başboğaoğlu, & Tolun, 2014). This recurrence suggests a possible founder effect or the existence of a mutational hotspot. *SRD5A3*-CDG is characterized by intellectual disability, ophthalmologic abnormalities including retinal dystrophy and optic atrophy, cerebellar involvement and ichthyosiform skin lesions. The observed phenotype in our patient is in line with previously described cases, with the characteristic triad of intellectual disability, ataxia, and visual impairment (Kamarus Jaman et al., 2021; Wheeler et al., 2016).

The *SNX14* splice-site variant in the family PKND6 is an example of the typical syndrome of cerebellar atrophy with progressive angular facial features and intellectual disability. (Akizu et al., 2015; Thomas et al., 2014) characterized this syndrome independently and demonstrated that *SNX14* encodes a sorting nexin involved in the function of lysosomes and autophagosomes. Loss-of-function mutations lead to lysosomal engorgement and impaired autophagosome clearance with cerebellar neurodegeneration. The clinical features we found in our patient, i.e., cerebellar atrophy, intellectual disability, and prominent facial features, are completely consistent with the established phenotype (Al-Hashmi, Mohammed, Al-Kathir, Al-Yarubi, & Scott, 2018; Sait, Moirangthem, Agrawal, & Phadke, 2022).

An in-frame deletion in *SLC6A8* in the family PKND8 causes creatine transporter deficiency, an X-linked disorder with cerebral creatine deficit, intellectual disability, speech delay and behavioral abnormalities. This disorder is the second most common cause of X-linked intellectual disability (after Fragile X syndrome), being found in 2% of males with unexplained intellectual disability (Salazar et al., 2020). Our patient presents with severe speech delay and behavioral issues, as presented by (Wang et al., 2018) and (Dunbar, Jaggumantri, Sargent, Stockler-Ipsiroglu, & van Karnebeek, 2014).

4.2 Clinical Implications and Future Perspectives

The identification of pathogenic variants in each of the four families highlights the clinical relevance of whole-exome sequencing (WES) as a primary mode of diagnostic testing in the neurodevelopmental disorder (NDD) in consanguineous groups. An accurate molecular diagnosis is the basis of refined genetic counselling, allows prenatal diagnosis in later pregnancies, and in some cases provides the basis of therapeutic intervention. As an illustration, patients with *SLC6A8* mutations may potentially be useful with the help of creatine supplementation, but the effectiveness of this treatment has not yet been proven beyond any doubt (Salazar et al., 2020).

5. Conclusion

To conclude, we have found that consanguineous families in Pakistan have pathogenic variants of five genes involved in NDDs. Our findings broaden the mutational spectrum of known NDD genes, such as *ASPM*, *SRD5A3*, *SNX14*, and *SLC6A8*. It is important to note that we found a novel variant in *SLC6A8* gene in hemizygous X-linked pattern with NDDs. The article emphasizes the significance of WES in the context of consanguinity populations, both in diagnostic and novel gene discovery, and contributes to the growing knowledge on the genetic architecture of neurodevelopmental disorders.

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Conflict of Interest

The authors declare no conflicts of interest.

References

1. Abdel-Hamid, M. S., Ismail, M. F., Darwish, H. A., Effat, L. K., Zaki, M. S., & Abdel-Salam, G. M. (2016). Molecular and phenotypic spectrum of ASPM-related primary microcephaly: Identification of eight novel mutations. *American Journal of Medical Genetics Part A*, 170(8), 2133-2140.
2. Akizu, N., Cantagrel, V., Zaki, M. S., Al-Gazali, L., Wang, X., Rosti, R. O., . . . Vaux, K. K. (2015). Biallelic mutations in SNX14 cause a syndromic form of cerebellar atrophy and lysosome-autophagosome dysfunction. *Nature genetics*, 47(5), 528-534.
3. Al-Hashmi, N., Mohammed, M., Al-Kathir, S., Al-Yarubi, N., & Scott, P. (2018). Exome sequencing identifies a novel sorting nexin 14 gene mutation causing cerebellar atrophy and intellectual disability. *Case Reports in Genetics*, 2018(1), 6737938.
4. Bennet, R. (1995). Recommendations for Standardized Human Pedigree Nomenclature. Pedigree in Human Genetics. *J Genetic Counseling*, 4, 4.
5. Cantagrel, V., Lefeber, D. J., Ng, B. G., Guan, Z., Silhavy, J. L., Bielas, S. L., . . . Swiezewska, E. (2010). SRD5A3 is required for converting polyprenol to dolichol and is mutated in a congenital glycosylation disorder. *Cell*, 142(2), 203-217.
6. Dunbar, M., Jaggumantri, S., Sargent, M., Stockler-Ipsiroglu, S., & van Karnebeek, C. D. (2014). Treatment of X-linked creatine transporter (SLC6A8) deficiency: systematic review of the literature and three new cases. *Molecular Genetics and Metabolism*, 112(4), 259-274.
7. Kamarus Jaman, N., Rehisi, P., Henderson, R. H., Löbel, U., Mankad, K., & Grunewald, S. (2021). SRD5A3-CDG: emerging phenotypic features of an ultrarare CDG subtype. *Frontiers in Genetics*, 12, 737094.
8. Kara, B., Ayhan, Ö., Gökçay, G., Başboğaoğlu, N., & Tolun, A. (2014). Adult phenotype and further phenotypic variability in SRD5A3-CDG. *BMC medical genetics*, 15(1), 10.
9. Létard, P., Drunat, S., Vial, Y., Duerinckx, S., Ernault, A., Amram, D., . . . Ceulemans, B. (2018). Autosomal recessive primary microcephaly due to ASPM mutations: An update. *Human mutation*, 39(3), 319-332.
10. Nicholas, A. K., Khurshid, M., Désir, J., Carvalho, O. P., Cox, J. J., Thornton, G., . . . Verloes, A. (2010). WDR62 is associated with the spindle pole and is mutated in human microcephaly. *Nature genetics*, 42(11), 1010-1014.
11. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., . . . Spector, E. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine*, 17(5), 405-423.
12. Sait, H., Moirangthem, A., Agrawal, V., & Phadke, S. R. (2022). Autosomal recessive spinocerebellar ataxia-20 due to a novel SNX14 variant in an Indian girl. *American Journal of Medical Genetics Part A*, 188(6), 1909-1914.
13. Salazar, M. D., Zelt, N. B., Saldivar, R., Kuntz, C. P., Chen, S., Penn, W. D., . . . Schleich, J. P. (2020). Classification of the molecular defects associated with pathogenic variants of the SLC6A8 creatine transporter. *Biochemistry*, 59(13), 1367-1377.
14. Srivastava, S., Love-Nichols, J. A., Dies, K. A., Ledbetter, D. H., Martin, C. L., Chung, W. K., . . . Prock, L. (2019). Meta-analysis and multidisciplinary consensus statement: exome sequencing is a first-tier clinical diagnostic test for individuals with neurodevelopmental disorders. *Genetics in Medicine*, 21(11), 2413-2421.

15. Thomas, A. C., Williams, H., Setó-Salvia, N., Bacchelli, C., Jenkins, D., O’Sullivan, M., . . . Chanudet, E. (2014). Mutations in SNX14 cause a distinctive autosomal-recessive cerebellar ataxia and intellectual disability syndrome. *The American Journal of Human Genetics*, 95(5), 611-621.
16. Wang, Q., Yang, J., Liu, Y., Li, X., Luo, F., & Xie, J. (2018). A novel SLC6A8 mutation associated with intellectual disabilities in a Chinese family exhibiting creatine transporter deficiency: case report. *BMC medical genetics*, 19(1), 193.
17. Wheeler, P. G., Ng, B. G., Sanford, L., Sutton, V. R., Bartholomew, D. W., Pastore, M. T., . . . Nickerson, D. A. (2016). SRD5A3-CDG: Expanding the phenotype of a congenital disorder of glycosylation with emphasis on adult onset features. *American Journal of Medical Genetics Part A*, 170(12), 3165-3171.