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Podcast

Expanding the phenotypic spectrum of *RNASEH2B* mutations: A new case of pure hereditary spastic paraplegia and a systematic review

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Keywords
Aicardi-Goutières Syndrome; Phenotypic Heterogeneity; Whole Exome Sequencing

Abstract

Background: Pathogenic variants in the *RNASEH2B* gene have been linked to Aicardi-Goutières syndrome type II (AGS-II), an early-onset encephalopathy that exhibits phenotypic overlaps with other neurodegenerative diseases, such as hereditary spastic paraplegia (HSP). A poor genotype-phenotype correlation, inconsistent findings in biomarker results of patients, and the challenge of distinguishing AGS-II from HSP underscore the necessity of performing comprehensive studies to address current difficulties in *RNASEH2B*-related cases. Here, through a detailed case report and comprehensive systematic review, we highlight clinical heterogeneity of *RNASEH2B*-related neurodegenerative cases and support the current view of considering *RNASEH2B* as an HSP-causing gene.

Methods: Using whole exome sequencing (WES), we identified an *RNASEH2B* variant, c.529G>A (p.Ala177Thr), in an Iranian patient suspected of having HSP, a mutation commonly reported in AGS-II. In contrast to AGS-II, clinical studies of the Iranian case were dominated by non-progressive HSP. A subsequent Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)-guided review of *RNASEH2B*-related neurodegenerative disorders identified 49 relevant cases from 349 studies, revealing a variable spectrum of phenotypes.

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Results: These phenotypes were classified into three overlapping categories: "*RNASEH2B*-related AGS", "atypical AGS-II", and "*RNASEH2B*-related HSP". 95 cases were diagnosed as *RNASEH2B*-related AGS or atypical AGS-II; six were classified as *RNASEH2B*-related HSP. One case was asymptomatic, and another involved intrauterine fetal death.

Conclusion: The current study highlights the expanding phenotypic spectrum of *RNASEH2B* mutations, emphasizing their potential to manifest as isolated pure HSP (pHSP) rather than classical AGS. This study underscores the importance of raising clinical awareness and incorporating genetic testing, particularly for atypical *RNASEH2B* cases.

Introduction

Aicardi-Goutières syndrome (AGS) is a rare and genetically heterogeneous encephalopathy caused by mutations in nine genes, including *TREX1*, *SAMHD1*, *ADAR*, *IFIH*, *LSM11*, *RNU7-1*, *RNASEH2A*, *RNASEH2B*, and *RNASEH2C*. The last three genes, *RNASEH2A-C*, encode different subunits of the ribonuclease H2 (RNase H2) enzyme, which recognizes and cleaves ribonucleic acid (RNA) strands in RNA/deoxyribonucleic acid (DNA) duplexes.[1,2] *RNASEH2B* is the most frequently mutated gene in patients with AGS, causing AGS type II (AGS-II). Phenotype complexity of *RNASEH2B* mutations may also be observed among the affected individuals in the same family.[3] Spasticity, psychomotor retardation, microcephaly, epilepsy, dystonia, and intellectual disability are prevalent clinical features in patients with AGS-II, which may also manifest in other neurodegenerative disorders.[4-8] Interestingly, in addition to these clinical overlaps, mutations in *RNASEH2B* have also been rarely reported in patients with suspected hereditary spastic paraplegia (HSP).[9-12]

Genetically, HSP is very heterogeneous, with more than 100 loci/88 causative genes and all modes of inheritance.[4] The prevalence of HSP is estimated to be 3-10 per 100000 in most populations, making HSP the second most common motor neuron disease (MND).[13] Clinically, HSPs are classified as pure or complex forms. Lower limb spasticity, subtle sensory signs, and bladder involvement are the hallmarks of pure forms. In contrast, complex forms are more complicated and also associated with additional neurological and non-neurological features, including cerebellar dysfunction, cognitive impairment, peripheral neuropathy, and orthopedic abnormalities.[5] Due to the phenotypic complexity and locus heterogeneity, the diagnosis of HSP is challenging.

The challenge becomes more complicated by considering HSP-mimicking conditions including AGS,[14,15] leukodystrophy (LD),[16] leukoencephalopathy,[17] neurodegeneration with brain iron accumulation (NBIA),[18] amyotrophic lateral sclerosis (ALS),[19] and coenzyme Q7 (COQ7)-associated primary coenzyme Q10 deficiency.[20,21] Recent advances in sequencing technologies have revolutionized the diagnostic yield of diseases with high clinical and genetic heterogeneity, such as MNDs. However, previous studies have demonstrated a diagnostic rate of 30% to 60% using next-generation sequencing (NGS) technologies for HSP.[10,22-25] Therefore, a precise diagnosis of the disease benefits significantly from a clear-cut description of the clinical features in all related patients, along with genetic analysis results and genotype-phenotype correlations.

In this regard, we reported the first Iranian *RNASEH2B*-related case who was diagnosed with HSP. Then, a systematic review of 103 *RNASEH2B*-related neurodegenerative cases was done, and the clinical, paraclinical, and molecular findings of these patients were described. Finally, we suggested that mutations in the *RNASEH2B* gene might cause a spectrum of disorders, including AGS-II, atypical AGS-II, and HSP phenotypes, and emphasized the role of *RNASEH2B* variants in the pathogenesis of HSP.

Materials and Methods

This research was verified by the ethics board of the University of Social Welfare and Rehabilitation Sciences, Tehran, Iran (IR.USWR.REC.1402.003), and performed according to the Declaration of Helsinki. Written informed consent for participation and publication of clinical details was obtained from the patient's legal guardian. All personal and medical information was anonymized to protect the patient's confidentiality. No harm was caused to the patient during the diagnostic process.

Subject

Clinical and paraclinical evaluations: A seven-year-old girl with a suspected HSP was referred to the Genetics Research Center at the University of Social Welfare and Rehabilitation Sciences for genetic analysis. The inheritance pattern of disease in the family seemed autosomal recessive since the proband, the only child in the family, was born to

asymptomatic consanguineous parents. Clinical and paraclinical evaluations, including magnetic resonance imaging (MRI), electromyography (EMG), nerve conduction study (NCS), electrocardiography (ECG), complete blood count (CBC), serum vitamin B12, serum T3, T4, and thyroid stimulating hormone (TSH) tests, serum lead, arterial blood gases (ABGs), and amino acid analysis in the cerebrospinal fluid (CSF), were performed for the proband.

Genetic analysis

Whole exome sequencing (WES): The DNA of the proband was extracted from peripheral blood using the conventional salting-out protocol. WES was performed by the Illumina HiSeq 4000 platform (Illumina). Data analysis covering quality control, indexing, sequence alignment against human reference genome University of California Santa Cruz (UCSC) National Center for Biotechnology Information (NCBI) Human Genome Build 37 (NCBI37)/Human Genome version 19 (hg19), variant calling, and annotation were performed using different bioinformatics tools including FastQC, Burrows-Wheeler Aligner (BWA), SAMTools, Picard, Genome Analysis ToolKit (GATK), and ANNOVAR. The process of analysis was explained in our antecedent investigations.[26] Preliminary filtering was done to identify all exonic, exonic splice, and splice site variants. Then variants that were synonymous with no effect on splicing were removed. Following that, variants with a reported minor allele frequency (MAF) more than 0.01 in the 1000 Genomes database (www.1000genomes.org), the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), the Genome Aggregation Database (http://genomad.broadinstitute.org/), the Healthy Exomes database (https://www.alzforum.org/exomes/hex), the Greater Middle East (GME) Variome Project (http://igm.ucsd.edu/gme/), or Iranome database (http://iranome.com/), or observed in the in-house exome data of 200 unrelated Iranians affected with non-neurological diseases were removed. The remaining variants were examined to identify those within any of known HSP or other neurodegenerative disease-causing genes.

The potential pathogenic effects of the variants on the encoded proteins were predicted using *in silico* tools including Polyphen2-HVAR (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT) (https://sift.bii.a-star.edu.sg/www/Extended_SIFT_chr_coords_submit.html), likelihood ratio test (LRT) (http://www.genetics.wustl.edu/jflab/lrt_query.html), Mutation Taster (http://www.mutationtaster.org/), Mutation Assessor (http://mutationassessor.org), FATHMM (http://fathmm.biocompute.org.uk/), Protein Variation Effect Analyzer (PROVEAN) (http://provean.jcvi.org/seq_submit.php), PANTHER (http://pantherdb.org/tools/csnpScoreForm.jsp?), Genomic Evolutionary Rate Profiling (GERP) (http://mendel.stanford.edu/sidowlab/downloads/gerp/index.html), Phylogenetic P-values (PhyloP) (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/phyloP44way), SIte-specific PHYlogenetic analysis (SiPhy), as well as Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu) webserver. The pathogenicity of variants was also assessed based on the American College of Medical Genetics and Genomics (ACMG) criteria.

Co-segregation of the candidate variant: Polymerase chain reaction (PCR) was used to amplify exon 7 of the *RNASEH2B* gene in the proband, which harbored the variant c.529G>A (p.Ala177Thr). The PCR product was sequenced using the Sanger method (Applied Biosystems, Foster City, CA, USA; Big Dye kit and Prism 3130 sequencer). Subsequently, the obtained sequence was analyzed by Sequencher 5.0 and compared with reference sequences available at NCBI (NM_024570.4, NP_078846.2). Subsequently, the variant was directly sequenced in the parents to conduct a co-segregation analysis of the variant with the disease status.

Systematic review

Search strategy: Here, we aim to study cases of *RNASEH2B*-related neurodegenerative disorders to describe the genetic and clinical heterogeneities within this group of diseases. To achieve this goal, we adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines,[27] ensuring that the systematic review process was rigorous and reproducible.

Identification: The process began with an extensive search of multiple bibliographical databases, including PubMed, Scopus, ScienceDirect, and Google Scholar, up to January 16, 2024. We used the search terms ((RNASEH2B) OR (Ribonuclease H2 Subunit B)) AND ((((Aicardi-Goutières syndrome) OR (AGS)) OR (hereditary spastic paraplegia)) OR (HSP)), ensuring that relevant studies were identified.

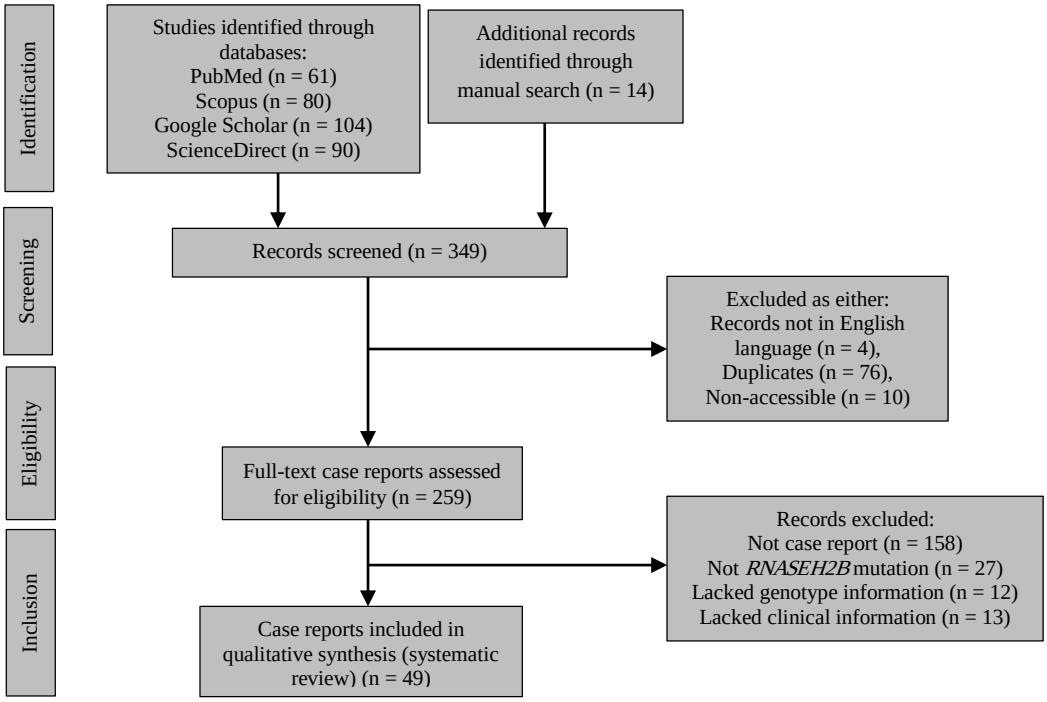

Figure 1. Flowchart of study selection for inclusion in the systematic review

Screening: After performing the initial search, we reviewed a total of 349 documents for title and abstract screening (Figure 1). Any non-English publications (4 studies), duplicates (76 studies), and non-accessible documents (10 studies) were excluded at this stage.

Eligibility: Following the exclusion of these irrelevant sources, we proceeded to the eligibility checking phase, where 259 documents were assessed for full-text review. During this stage, 210 documents were removed, including review articles, books/chapters, and functional studies (158 studies), cases of AGS without *RNASEH2B* mutations (27 studies), and AGS-II cases lacking sufficient clinical or genotype data (25 studies). Two independent investigators carried out the full-text evaluation, and any disagreements were discussed and resolved collaboratively.

Inclusion: Ultimately, 49 publications met the inclusion criteria for further analysis. All included records (49 publications: 103 *RNASEH2B*-related cases) studied patients with confirmed *RNASEH2B* mutations and clearly reported both genotype and phenotype information. Data extraction was performed systematically, reviewing each selected case's demographic, genetic, clinical, and paraclinical information to ensure that all relevant details were included (Supplementary Table S1).

Results

Clinical and paraclinical features of the Iranian case:

The proband was a 7-year-old girl affected by a neurological disease that started in early childhood. She was referred to us as a suspected HSP case. She was born to unaffected, distantly related consanguineous parents (Supplementary Figure S1) via normal pregnancy and delivery. Based on her mother's explanations, her development during infancy, in the first two years of life, was normal with the natural acquisition of motor milestones. She manifested her first sign at the age of 2 years when her parents noticed she walked on her toes and showed some degree of stiffness in both legs. Her condition began to get worse gradually, with her gait becoming more effortful.

Due to the shortening of the Achilles tendon, she underwent surgery for tendon release and elongation last year. On neurologic examination, she showed a mild increase in the jaw reflex. Fundoscopic examination and assessment of eye movements revealed no abnormalities. The muscle tone of the lower limbs was spastic, while the upper limbs had a normal tone. Spasticity of the lower limbs was accompanied by a decrease in force (3/5 score for feet dorsiflexion and 4/5 for feet plantar flexion). Muscle stretch reflexes were evaluated to be 3/4 for both upper and lower limbs. Plantar reflexes were upward bilaterally (Babinski sign). She showed mild dystonic posture in the right hand and had severe spasticity of the adductor muscles and a steppage gait.

Cerebellar evaluations were normal. She had urinary urgency while laughing, and had no sensory symptoms, seizures, cognitive deterioration, ophthalmic or auditory problems, dysarthria, dysphagia, facial dysmorphism, or microcephaly. No sign of abnormal pigmentation or a lesion on her skin was detected. Her EMG, NCS, and ECG had normal results. Her brain MRI only showed thinning of corpus callosum (TCC). Routine lab tests, including vitamin B12, T3, T4, and TSH hormones, and amino acid analysis were normal according to her age.

WES and bioinformatics analyses results: After variant filtering according to the mentioned criteria, a homozygous variant – c.529G>A (p.Ala177Thr) – in the *RNASEH2B* gene (NM_024570.4) was identified in the proband. This known variant was in heterozygous state in her parents; it had been previously reported in both patients with HSP[9-12] and patients with AGS-II,[3,28,29] and was classified as pathogenic or likely pathogenic based on the adjusted or default ACMG criteria, respectively. The variant presented a low frequency or absence in the public genome databases and it was predicted to be a deleterious or damaging variant based on the *in silico* tools described in the Materials and Methods section. Evolutionary assessments by GERP++, PhyloP, and SiPhy showed that this nucleotide was highly conserved. The CADD score was 19.86.

Literature review

Overview of included cases and studies: A total of 103 *RNASEH2B*-related neurodegenerative patients from 49 records were included in this survey for phenotype and genotype descriptions. Generally, positive consanguinity status had been specified only in 17 out of 63 reported cases (not mentioned for 40 cases). The mean age at onset (AAO) of the disease, available for 69 cases, was 1.03 ($\pm$ 2.29) years. Except for three cases (#36, #37, #50; Supplementary Table S1), the clinical symptoms of all individuals were manifested in the first two years of life. Precisely, the AAO of the majority of cases (51 cases) was within the first year of life and 18 cases presented 1 year < AAO < 2 years.

RNASEH2B causative variants: The *RNASEH2B* gene is composed of 11 exons and encodes a 312-amino acid protein (Figure 2). To date, 60 mutations in *RNASEH2B* have been reported in the Human Gene Mutation Database (HGMD) (Professional 2023.4: http://www.hgmd.cf.ac.uk/ac/index.php) and literature (Figure 2). Although these mutations have been scattered throughout the gene, a hotspot exon, exon 7, was detected. It seems the types and positions of these mutations can affect the structure, biosynthesis, and function of the RNASEH2B protein and, consequently, the clinical features of the patients. However, their exact biological mechanisms still remain unclear. Among the 60 reported mutations, only 22 distinct mutations were identified in the 49 included publications. Again, the majority of the 103 included cases carried a missense mutation in exon 7 (153 mutated alleles out of 203, 75.3%), exon 5 (23 mutated alleles, 11.3%), or intron 4 (9 mutated alleles, 4.4%) (Figure 2). The most common pathogenic variant in the included cases (80 out of 103 cases, 77.6%) was c.529G>A (p.Ala177Thr). Indeed, the recurrent variant c.529G>A in a pan-ethnic cohort of patients demonstrates this variant is a hotspot rather than a founder mutation. In the protein level, most of the included mutations (181 of 203 mutated alleles, 89.1%) have been located in the RNase_H2-Ydr279 domain of the protein. Totally, based on all reported mutations in HGMD, 15 out of 33 missense mutations in the *RNASEH2B* genes (45.4%) have been located in the RNase_H2-Ydr279 domain of the protein (Figure 2).

Clinical and paraclinical findings: Totally, ninety-five cases were diagnosed as AGS-II or atypical AGS-II, while six cases presented with an HSP phenotype. Due to insufficient data, individual #41, an asymptomatic case, and case #89, which involved intrauterine fetal death, have been excluded from our clinical and paraclinical analysis. Among the 101 remaining *RNASEH2B*-neurodegenerative cases with available clinical information, spasticity, developmental delay, and microcephaly were the most frequent clinical features observed in 73 (72.2%), 60 (59.4%), and 33 (32.6%) cases, respectively. Dystonia, intellectual disability, and seizure were the next common features. The distribution of additional clinical features is illustrated in figure 3 [clinical features of AGS-II/atypical AGS-II, and pure HSP (pHSP) cases were presented in two parallel columns]. White matter (WM) abnormalities and brain atrophic changes were the most common features observed in the MRI of 63 and 35 cases, respectively.

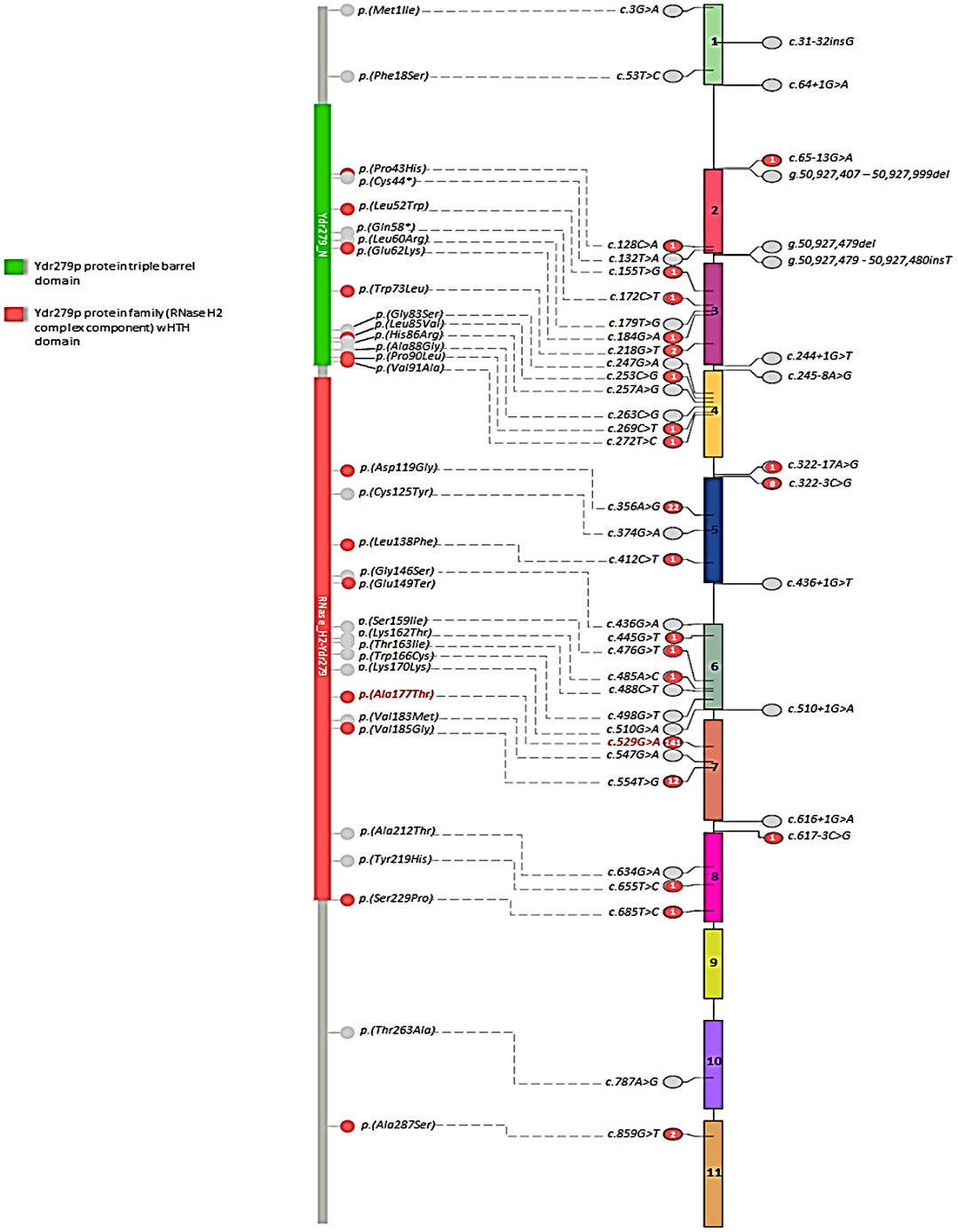

Figure 2. Schematic representation of the *RNASEH2B* gene (right) and protein domains (left), with the position of identified mutations. The circles (both gray and red) represent the position of all reported mutations in the *RNASEH2B* gene based on the Human Gene Mutation Database (HGMD; professional 2023.4) and literature until January 16, 2024. Red circles are 22 mutations found in the 103 included cases of this study. Numbers within red circles represent the number of mutated alleles identified. (wHTH: winged helix-turn-helix)

Moreover, the computed tomography (CT) scan results of affected individuals reported brain calcification as the most common feature that was observed in 44 cases. Despite the limited number of patients with HSP, significant findings emerge when contrasting six patients with *RNASEH2B*-related HSP against the remaining 95 *RNASEH2B*-related cases.

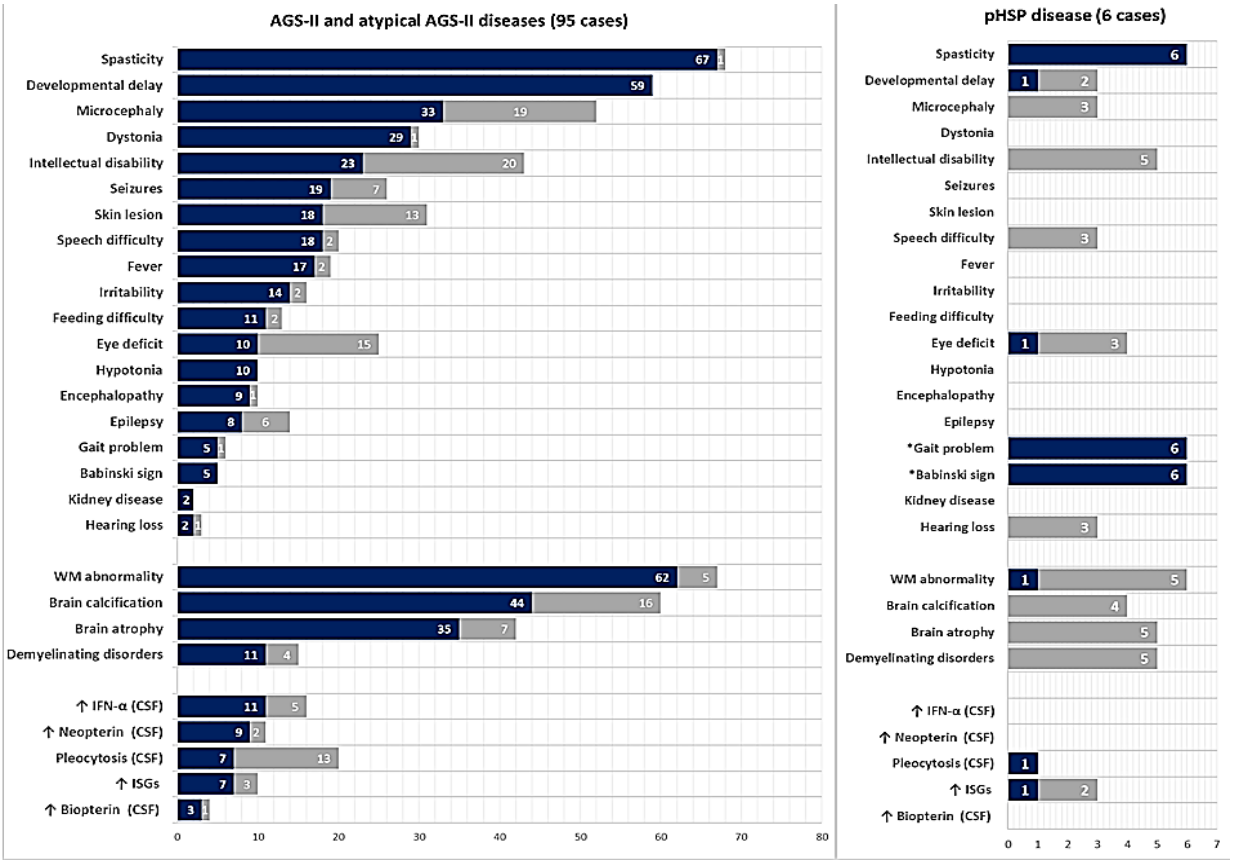

Figure 3. Distribution of clinical and paraclinical features in 101 *RNASEH2B*-related neurodegenerative cases. "*RNASEH2B*-related Aicardi-Goutières syndrome (AGS)" and "atypical AGS-type II (AGS-II)" (95 cases) and "*RNASEH2B*-related hereditary spastic paraplegia (HSP)" (6 cases) have been shown on the left and right, respectively. *Babinski's sign and gait problems were likely present in all 6 patients with pure HSP (pHSP), but the specific terms "Babinski" and "gait" were not explicitly mentioned in three of those cases. Skin lesions included chilblains, angiokeratomas, and erythematous lesions. Positive and negative findings are presented by blue and gray color bars (with numbers inside the bars), respectively. NB: We had access to clinical data from 101 cases, and neuroimaging results from 88 cases were available for study. (WM: White matter; CSF: Cerebrospinal fluid; IFN-α: Interferon-alpha; ISG: Interferon-stimulated gene)

While spasticity is reported in all patients with HSP, brain atrophy and calcification were entirely absent in these patients and have been exclusively observed in AGS-II cases (Figure 3).

Discussion

AGS-II or HSP?: The initial reports only linked *RNASEH2B* mutations to AGS-II disease. However, the clinical and paraclinical profile of our patient with the *RNASEH2B* mutation does not completely align with AGS-II disease. Our case manifested lower limb spasticity, which is the predominant symptom of HSP and has been reported in ~ 70% of *RNASEH2B*-related neurodegenerative cases. Moreover, similar to some HSP cases, our case demonstrated neurogenic bladder and TCC in her brain MRI.[19] However, microcephaly, cognitive impairment, skin lesions, seizure, cerebral atrophy, WM abnormality, and brain calcification, which have been reported for AGS-II[15] (Figure 3), were not observed in our case. Since our case presented symptoms resembling pHSP, we investigated all documented cases who carried a mutation in the *RNASEH2B* gene to deepen our understanding of outcomes associated with *RNASEH2B* variants. Our systematic review revealed that among 103 *RNASEH2B*-neurodegenerative cases, six individuals from four studies manifested clinical presentations of pHSP (Table 1). Except for one compound heterozygote case, all *RNASEH2B*-related HSP cases, including ours harbored the same homozygous c.529G>A (p.Ala177Thr) variant.[9-12] Interestingly, the c.529G>A mutation is observed not only in *RNASEH2B*-related HSP cases but also in a significant percentage of patients with AGS-II.

Table 1. Molecular, clinical, and paraclinical features of individuals with *RNASEH2B*-related hereditary spastic paraplegia (HSP) disease

Reference	Crow et al.[12]			Travaglini et al.[10]	Spagnoli et al.[11]	Agarwal et al.[9]	Present study
	Patient 1	Patient 2	Patient 3				
Gene name	*RNASEH2B*	*RNASEH2B*	*RNASEH2B*	*RNASEH2B*	*RNASEH2B*	*RNASEH2B*	*RNASEH2B*
Variant in cDNA	c.529G>A	c.529G>A	c.529G>A	c.529G>A	c.529G>A	c.529G>A, c.617-3C>G	c.529G>A
Variant in protein	p.(Ala177Thr)	p.(Ala177Thr)	p.(Ala177Thr)	p.(Ala177Thr)	p.(Ala177Thr)	p.(Ala177Thr), mis-splicing	p.(Ala177Thr)
Zygosity	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Compound heterozygous	Homozygous
Gender	Girl	Girl	Boy	Girl	Boy	Girl	Girl
Consanguinity	-	-	-	NA	-	-	+
Ethnic groups	Egyptian	Egyptian	North African	NA	NA	Indian	Iranian
Classification	Pure HSP	Pure HSP	Pure HSP	Pure HSP	Pure HSP	Pure HSP	Pure HSP
Age at onset (month)	12	24	23	12	Infancy	24	24
Gait problem	Scissoring gait	Scissoring gait	Scissoring gait	NA	NA		Steppage gait
Babinski sign	+	+	+	NA	NA		+
Lower limb weakness	+	+	NA	NA	NA		-
Toe walking	NA	NA	NA	+	NA		+
Lower limb spasticity	+	+	+	+	+	+	+
Motor delay	NA	NA	NA	NA	+		-
Dystonia	NA	NA	NA	NA	NA		+
Speech difficulties	-	-	-	NA	NA		-
Intellectual disability	-	-	-	NA	-	-	-
Ocular abnormalities	Blindness (left eye), optic atrophy	-	-	NA	-	-	-
Hearing impairment	-	-	-	NA	NA	-	-
CSF analysis	NA	NA	Lymphocytosis	NA	NA	NA	NA
ISGs	Normal	Increased	Normal	NA	NA	NA	NA
Brain MRI	Normal	Normal	Nonspecific high signal on T2-weighted imaging, dilatation of lateral ventricles	Normal	Normal	Normal	TCC
CT scan	Normal	Normal	Normal	NA	Normal	NA	NA
Other	Recurrent falls, mild increase in tone with brisk reflexes in upper limbs	Recurrent falls, mild increase in tone with brisk reflexes in upper limbs	Increased reflexes, clonus; the pregnancy was complicated by gestational diabetes		Slight asymmetry of the optic papillae excavation	Urinary urgency and occasional urge incontinence, exaggerated lower limb reflexes	CBC: Lymphocytosis, urinary urgency during laughing, stiffness of legs, mild jaw jerk increment

cDNA: Complementary deoxyribonucleic acid; CSF: Cerebrospinal fluid; ISG: Interferon-stimulated gene; MRI: Magnetic resonance imaging; CT: Computed tomography; TCC: Thinning of corpus callosum; HSP: Hereditary spastic paraplegia; CBC: Complete blood count; NA: Not available

The existence of such phenotypic heterogeneity might be in favor of a similar pathophysiology for these diseases. Thus, we propose that, genetically, all of the 103 included cases in this study who carried a mutation in *RNASEH2B* can be classified into one category named *RNASEH2B*-related neurodegenerative disorder. However, the clinical and paraclinical profiles of these 103 cases show a wide and variable spectrum of symptoms, which can be classified into three sub-categories: "*RNASEH2B*-related AGSs", "*RNASEH2B*-related HSPs", and "atypical AGS-II" (Supplementary Figure S2).

***RNASEH2B* mutations: Expanding the clinical spectrum beyond AGS-II**

RNASEH2B-related HSP cases exhibited milder clinical manifestations compared to classical AGS-II (Figure 3 and Table 1). The mean AAO of these pHSP cases was 1.58 (± 0.53) years, and spasticity, Babinski sign, and gait problems were their most common features. The remaining 95 patients were initially diagnosed with AGS-II and their average AAO was 0.99 (± 2.37) years. The typical HSP features, including spasticity, Babinski sign, and gait problem, were observed in 70.5%, 5%, and 5% of these cases, respectively. Developmental delay (62%), microcephaly (34%), WM abnormality (65%), brain calcification (46%), dystonia (30%), intellectual disability (24%), skin lesions (18%), and raised interferon-alpha (IFN-α) concentration (11%) were the most common clinical and paraclinical features in these *RNASEH2B*-related AGS cases. However, these features were either rare or absent in *RNASEH2B*-related HSP. Interestingly, a detailed evaluation of the clinical and paraclinical features of 95 AGS-II cases demonstrated that some individuals might not qualify for a definite diagnosis of AGS-II due to the absence of the typical clinical and paraclinical hallmarks of the disease. For example, atypical AGS-II cases #7, #37, #45, #52, #56, and #86[30-35] (Supplementary Table S1) lacked typical AGS-II or HSP (Supplementary Figure S2) features that placed them in an intermediate phenotype between AGS-II and HSP.

***RNASEH2B* mutations: Genotype-phenotype correlation**

The RNase H2 subunit B (H2B) protein, encoded by RNASEH2B, has two domains: The Ydr279p protein triple barrel domain (34aa-92aa) and the Ydr279p protein family winged helix-turn-helix (wHTH) domain (95aa-228aa). Structurally, this protein consists of seven β strands and one α helix that cleaves RNA in DNA: RNA hybrids.[1,2,36] The C-terminal tail of RNASEH2B consists of a proliferating cell nuclear antigen (PCNA)-interacting protein-box (PIP-box) motif, residues 294-301, that regulates the interaction of the RNase H2 complex with PCNA[37] as well as its localization to replication foci.[38] The most common pathogenic mutation of the *RNASEH2B* gene was c.529G>A (141 of 203 mutant alleles, 69.4%). Previous structural and biochemical studies demonstrated that the c.529G>A (p.Ala177Thr) mutation was located close to the H2B/RNase H2 subunit C (H2C) interface, disrupted the interaction between an RNASEH2B α-helix and the RNASEH2C kinked helix, and finally reduced RNase H2 complex stability.[39,40] Ultimately, in this study, we sought to realize whether the differences in *RNASEH2B* mutations might shed light on the underlying mechanisms of the clinical features. But apparently, it is difficult to find a direct correlation between the location of the mutations and clinical features. Most mutations (89.1%) have been located in the RNase_H2-Ydr279 domain of the protein (Figure 2) and mutations in this domain were not phenotype-specific. Typically, patients with *RNASEH2B* mutations present a combination of several clinical and paraclinical features, and they manifest variable expressivity and also phenotypic heterogeneity even within a family with the same mutation.[33,35] These complexities made it very difficult to find an obvious phenotype-genotype correlation.

***RNASEH2B*-related neurodegenerative disorder: Diagnostic challenges**

Increasing number of the *RNASEH2B*-related cases may potentially even expand the spectrum of phenotypes linked to the *RNASEH2B* mutations. Further adding to this complexity is that a specific mutation, c.529G>A, in *RNASEH2B* could result in phenotypic heterogeneity and the presentation of two apparently distinct disorders, AGS-II or HSP, suggesting the role of other genetics, epigenetics, or environmental factors. It seems, in addition to phenotypic heterogeneity and variable expressivity, c.529G>A is associated with incomplete penetrance, where the AAO of several affected individuals (#42, #44, #88 in Supplementary Table S1) was at birth,[3,41] while the individual #41 with the same genetic mutation is still asymptomatic at the age of 42 years old.[3] Elevated levels of IFN-α in CSF were also observed in 11 cases (serological data for 54 cases were not available). However, the

heterogeneity of the reported results and the documentation of normal levels of IFN-α activity in several cases (e.g., #7, #33, #52, #95, and #100; Supplementary Table S1) cast doubt on the diagnostic criterion of the expression levels of IFN-α as a sensitive biomarker for AGS-II disease.

Limitations: Finally, while our study contributes to expanding the clinical spectrum of *RNASEH2B* mutations, we clearly acknowledge the study's limitations. Firstly, the small sample size of *RNASEH2B*-related HSP cases limits the generalizability of our conclusions. Only six cases of *RNASEH2B*-related HSP have been documented in the literature, including the case described in this study, which restricts our ability to fully characterize the diverse manifestations of this disorder. Secondly, as the review is based on data extracted from previously published studies with differing methodologies, diagnostic criteria, and patient characteristics, certain limitations in consistency and comparability are acknowledged. Additionally, the absence of functional studies in our research means we cannot further discuss the underlying mechanisms of the c.529G>A mutation that drives HSP or AGS-II disorders. Future prospective studies incorporating functional validation and larger sample sizes will be essential to further elucidate the pathophysiological mechanisms behind *RNASEH2B*-related disorders.

Conclusion

In this study, a detailed evaluation of the affected Iranian individual presented the seventh report of the *RNASEH2B*-related HSP. The c.529G>A mutation identified in our case has been reported in all previously described *RNASEH2B*-related HSP cases. In comparison with *RNASEH2B*-related AGS-II, *RNASEH2B*-related HSP disease exhibited a milder phenotype with later onset in childhood. The clinical profile of individuals with *RNASEH2B*-related AGS-II disease encompassed a wide spectrum, involving both neurological and non-neurological symptoms. However, the presentation of *RNASEH2B*-related HSP cases was dominated by non-progressive spastic paraplegia. Furthermore, the specific mutation c.529G>A has been detected in both *RNASEH2B*-related AGS-II (67 out of 89 cases) and *RNASEH2B*-related HSP cases (6 out of 6 cases). This kind of genetic overlapping is not unique to this particular gene, and it has also been reported in *C19orf12*,[42] *COQ7*,[20,21] *GJC2*,[43] and many other genes. Therefore, we expect that the increasing reports of *RNASEH2B*-related HSP cases could establish *RNASEH2B* as an HSP-related gene in the expanding list of HSP-associated genes.

Conflict of Interests

The authors declare no conflict of interest in this study.

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