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Novel Digital Anomalies, Hippocampal Atrophy, and Mutations Expand the Genotypic and Phenotypic Spectra of *CNKSR2* in the Houge Type of X-Linked Syndromic Intellectual Development Disorder (MRXSHG)

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ABSTRACT

The Houge type of X-linked syndromic intellectual developmental disorder (MRXSHG) encompasses a spectrum of neurodevelopmental disorders characterized by intellectual disability (ID), language/speech delay, attention issues, and epilepsy. These conditions arise from hemizygous or heterozygous deletions, along with point mutations, affecting *CNKSR2*, a gene located at Xp22.12. CNKSR2, also known as CNK2 or MAGUIN, functions as a synaptic scaffolding molecule within the neuronal postsynaptic density (PSD) of the central nervous system. It acts as a link connecting postsynaptic structural proteins, such as PSD95 and S-SCAM, by employing multiple functional domains crucial for synaptic signaling and protein–protein interactions. Predominantly expressed in dendrites, CNKSR2 is vital for dendritic spine morphogenesis in hippocampal neurons. Its loss-of-function variants result in reduced PSD size and impaired hippocampal development, affecting processes including neuronal proliferation, migration, and synaptogenesis. We present 15 patients including three from the MENA (Middle East and North Africa), a region with no documented mutations in *CNKSR2*. Each individual displays unique clinical presentations that encompass developmental delay, ID, language/speech delay, epilepsy, and autism. Genetic analyses revealed 14 distinct variants in *CNKSR2*, comprising five nonsense, three frameshift, two splice, and four missense variants, of which 13 are novel. The

Abbreviations: ADHD, attention deficit hyperactivity disorder; ADOS, Autism Diagnostic Observation Schedule; AGA, appropriate for gestational age; ASD, autism spectrum disorder; BERA, Brainstem Evoked Response Audiometry; CADD, combined annotation dependent depletion; CBD, cytohesin binding domain; CNK2, connector enhancer of kinase suppressor of RAS-2; CNKSR2, connector enhancer of kinase suppressor of Ras-2; CSWS, continuous spikes and waves during sleep; CYTH2, Cytohesin-2; DEE-CSWS, developmental and epileptic encephalopathy linked to continuous spikes and waves during sleep; EAS, epilepsy-aphasia spectrum; EEG, Electroencephalogram; ERG, electroretinogram; ES, exome sequencing; ESES, electrical status epilepticus during sleep; EVS, Exome Variant Server; FISH, fluorescence in situ hybridization; FRAXA, Fragile X syndrome-A; GA, gestational age; GATK, Genome Analysis Toolkit; GTC, generalized tonic–clonic; HGMD, Human Gene Mutation Database; ID, intellectual disability; KO, knockout; MAF, minor allele frequency; MAGUIN, membrane-associated guanylate kinase-interacting protein; MAPK, mitogen-activated protein kinase; MENA, Middle East and North Africa; MRI, magnetic resonance imaging; MRXSHG, Houge type of X-linked syndromic intellectual developmental disorder; NCS, nerve conduction study; NDD, neurodevelopmental disorder; PEA, pulmonary edema assessment; PFC, palmar flexion crease; PH, pleckstrin homology; PSD, postsynaptic density; PSD-95, postsynaptic density 95; REELS, Receptive Expressive Emergent Language Scale; SAP, synaptic-associated protein; S-SCAM, synaptic scaffolding molecule; VEP, Visual Evoked Potentials; XLID, X-linked intellectual disability.

Sahand Tehrani Fateh and Afif Ben-Mahmoud contributed equally to this study.

For affiliations refer to page 18.

ACMG guidelines unanimously interpreted these 14 variants in 15 individuals as pathogenic, highlighting the detrimental impact of these *CNKSR2* genetic alterations and confirming the molecular diagnosis of MRXSHG. Importantly, variants Ser767Phe and Ala827Pro may lead to proteasomal degradation or reduced PSD size, contributing to the neurodevelopmental phenotype. Furthermore, these two amino acids, along with another two affected by four missense variants, exhibit complete conservation in nine vertebrate species, illuminating their crucial role in the gene's functionality. Our study revealed unique new digital and brain phenotype, including pointed fingertips (fetal pads of fingertips), syndactyly, tapering fingers, and hippocampal atrophy. These novel clinical features in MRXSHG, combined with 13 novel variants, expand the phenotypic and genotypic spectra of MRXSHG associated with *CNKSR2* mutations.

1 | Background

In Western countries, families with X-linked intellectual disability (XLID) are more common than those with autosomal dominant ID because asymptomatic female carriers often go unnoticed and have a greater chance of having children. Additionally, the higher prevalence of XLID in males compared to females has long led to the recognition of X-linked gene defects as significant contributors to ID (Ropers and Hamel 2005). XLID accounts for more than 16% of male ID cases (Stevenson and Schwartz 2009) and represents a group of disorders with early-onset neurodevelopmental delay (DD), resulting in significant impairments in intellectual functioning and adaptive behavior (Ropers and Hamel 2005). The X chromosome constitutes approximately 5% of the human genome and harbors 141 genes associated with ID (Neri et al. 2018). The Houge type of X-linked syndromic intellectual disability (MRXSHG, MIM 301008) represents a spectrum of neurodevelopmental disorders (NDDs), affecting both males and, to a lesser extent, females, and is characterized by ID, language/speech delay, attention problems, and epilepsy. This condition is associated with hemizygous or heterozygous deletions, as well as point mutations, which lead to the loss-of-function of CNKSR2 (connector enhancer of kinase suppressor of Ras-2, MIM 300724) at Xp22.12 (Damiano et al. 2017).

CNKSR2, also known as CNK2 (connector enhancer of kinase suppressor of RAS-2) or MAGUIN (membrane-associated guanylate kinase-interacting protein), is a synaptic molecule with multiple functional domains, including SAM, CRIC, PDZ, DUF1170, PH, and a PDZ-binding motif (Lim et al. 2014), each playing specific roles in cellular signaling and protein–protein interactions. CNKSR2 demonstrates structural conservation across species (Lanigan et al. 2003) and the four putative protein–protein binding domains, SAM, CRIC, PDZ, and PH are preserved in CNKSR families across species (Lanigan et al. 2003; Zieger et al. 2020). Initially identified as a binding partner for postsynaptic density 95 (PSD-95) and synaptic scaffolding molecule (S-SCAM) to form a complex (Yao et al. 1999), CNKSR2's interaction with PSD-95 and S-SCAM is mediated by its PDZ-binding motif and the corresponding PDZ domains of PSD95 or S-SCAM. Comprising four amino acids—ETHV—derived from residues 1031–1034, this PDZ-binding motif is located at the tip of CNKSR2's C-terminus (Figure 1). Effectively, CNKSR2 acts as a shared ligand for both PSD-95 and S-SCAM (Yao et al. 1999).

This complex assembles the components of synaptic junctions (Yao et al. 1999) and plays a crucial role in RAS/MAPK signaling, mediating essential neurodevelopmental processes, including neuronal proliferation, migration, differentiation, and apoptosis, as well as RAS-mediated synaptic formation (Aypar, Wirrell, and Hoppman 2015; Vaags et al. 2014; Houge, Rasmussen, and Hovland 2011; Hu et al. 2016).

Predominantly expressed in key brain regions such as the hippocampus, amygdala, and cerebellum (Yao et al. 1999), CNKSR2 functions as a scaffold/adaptor within the neuronal PSD of the central nervous system, potentially linking postsynaptic structural proteins such as PSD95. Additionally, CNKSR2, a synaptic membrane-associated protein, is specifically expressed in neurons, particularly within dendrites, and is essential for dendritic spine morphogenesis in hippocampal neurons (Lim et al. 2014). Its localization extends to postsynaptic sites, and its loss results in a reduced size of the PSD in neurons (Zieger et al. 2020). Moreover, CNKSR2 stimulates the mitogen-activated protein kinase (MAPK) pathway, that is important for the growth of neuronal dendrites. Consequently, alterations in the anchoring apparatus of the postsynaptic complex may result in NDDs (Zieger et al. 2020). A Cnksr2 knockout (KO) mouse line was recently created, showing increased neural activity and spontaneous electrographic seizures. Moreover, these mice displayed heightened anxiety, learning and memory impairments, and a progressive loss of ultrasonic vocalizations (Erata et al. 2021). These phenotypes recapitulate symptoms observed in epilepsy-aphasia spectrum (EAS) and MRXSHG patients, making the KO mice valuable for studying the pathophysiology of these conditions.

Within the ever-expanding and diverse phenotypic spectrum of MRXSHG, we conducted a comprehensive examination of the detailed phenotypes of 15 patients (Section 2). Additionally, we sought to determine potential genotype–phenotype correlations by comparing their phenotypes not only with each other but also with those of patients carrying both similar and different *CNKSR2* mutations (Tables 1 and Table S1). Our primary goal was to establish any discernible connections between the specific *CNKSR2* variants and the observed phenotypic features, ultimately contributing to our understanding of how *CNKSR2* alterations can lead to a wide range of NDDs.

2 | Results

2.1 | Clinical Evaluation of Human Subjects

2.1.1 | Subject 1 (c.2479G>C, p.Ala827Pro)

In a Yemeni family, five children were born to two healthy, unrelated parents, both of whom are college graduates. Among these children, the youngest is a male who has been diagnosed

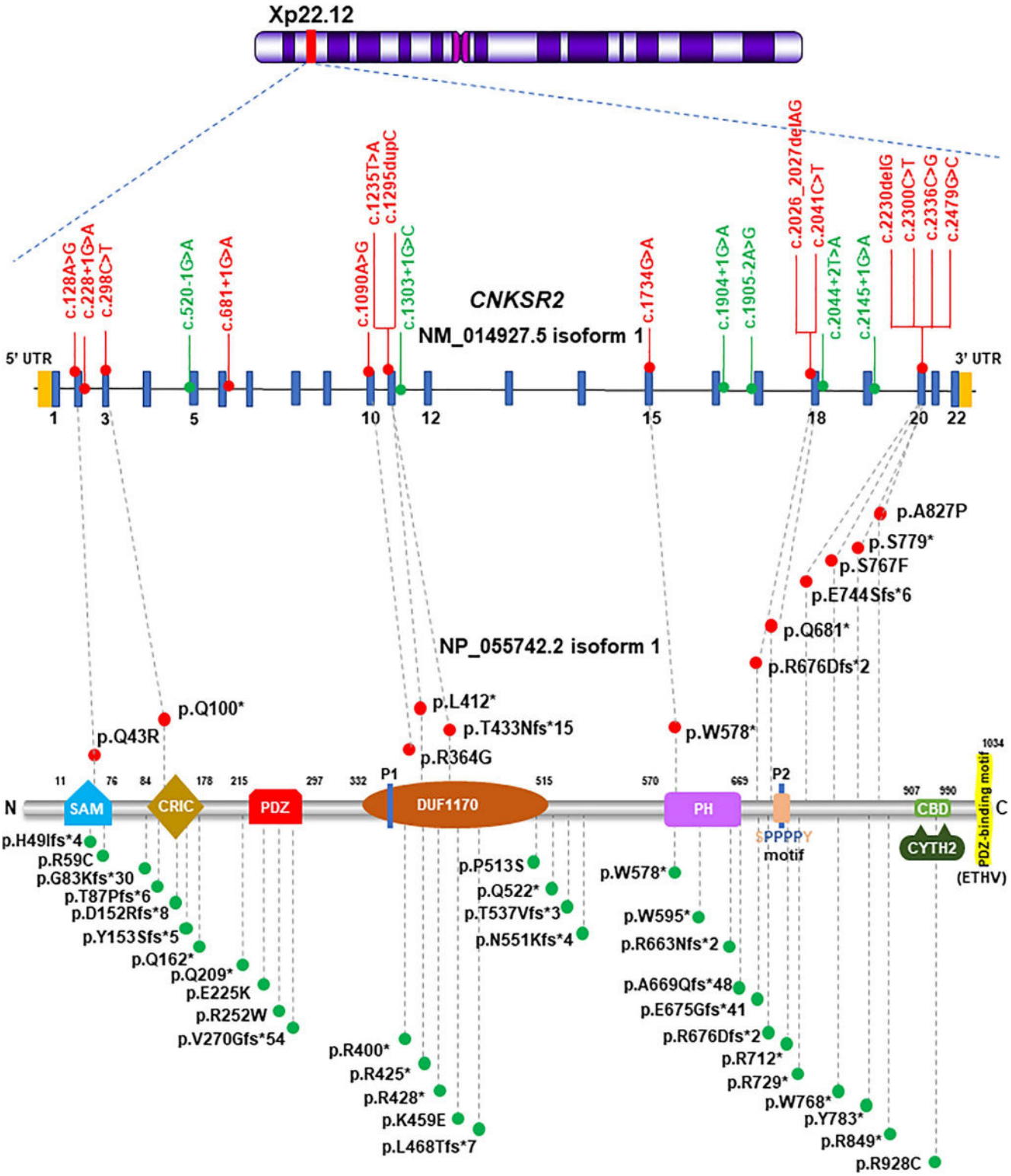

FIGURE 1 | Localization of novel and reported mutations in *CNKSR2* at Xp22.12. The upper panel illustrates the 13 intragenic mutations identified in this study, represented by read balls, along with six reported splice mutations, represented by green balls, within their respective exons and introns of *CNKSR2* isoform 1 (NM_014927.5). Exons are represented in blue, connected by a horizontal black line representing introns, and the yellow boxes depict the UTRs. In the lower panel, we present the domain structure of CNKSR2 protein isoform 1 (NP_055742.2) with dotted black lines indicating matching mutations and their corresponding CNKSR2 amino acid residue numbers. Below the protein structure of CNKSR2, the 32 reported mutations are represented by green balls. Dotted black lines connect these mutations to the corresponding protein domains. P1: The first short proline-rich motif (PPPP, aa 354–357), P2: The second short proline-rich motif (PPPP, aa 703–706), CBD: Coiled-coil Cytosin Binding Domain (aa 907–990), CYTH2: Cytohesin2, PDZ-binding motif: (ETHV, aa 1031–1034).

TABLE 1 | Clinical features of 15 subjects with intragenic mutations in *CNKSR2* (NM_014927.5).

Subjects ID	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8
Nucleotide change NM_014927.5	c.2479G>C	c.1734G>A	c.2300C>T	c.2026_2027delAG	c.2041C>T	c.2230delG	c.2336C>G	c.1295dup
Exon/intron	Exon 20	Exon 15	Exon 20	Exon 18	Exon 18	Exon 20	Exon 20	Exon 11
Sex	M	M	M	M	M	M	F	M
Current age	13 years	8 years	25 years	12 years	3 years 6 months	57 years	6 years	17 years
Effect on protein NP_055742.2	p.Ala827Pro	p.Trp578Ter	p.Ser767Phe	p.Arg676Asp*fs**2	p.Gln681Ter	p.Glu744Serfs*6	p.Ser779Ter	p.Thr433Asnfs*15
Genomic position HG 19	g.21627522G>C	g.21609216G>A	g.21627343C>T	g.21619449_21619450del	g.21619464C>T	g.21627273del	g.21627379C>G	g.21550177dup
ACMG classification	PS4, PM2, PM3, PP1, PP2, PP3, PP4 pathogenic	PVS1, PS1, PS2, PS4, PM2, PP3 pathogenic	PS4, PM2, PM3, PP1, PP2, PP3, PP4 pathogenic	PVS1, PS2, PS4, PM2 pathogenic	PVS1, PS4, PM2, PM3, PP1, PP3, PP4 pathogenic	PVS1, PS4, PM2, PM3, PP1, PP4 pathogenic	PVS1, PS2, PS4, PM2, PP3 pathogenic	PVS1, PS4, PM2, PM3, PP1, PP4 pathogenic
MAF (gnomAD)	0	0	0	0	0	0	0	0
M-CAP SCORE	N/A	N/A	Possibly pathogenic	N/A	N/A	N/A	N/A	N/A
CADD score	19.86	37	27.5	N/A	40	N/A	37	N/A
Inheritance	Maternal	*de novo*	Maternal; three maternal uncles with id and epilepsy	*de novo*	Maternal	Maternal; affected brother not tested	*de novo*	Maternal; mother observed with hypertelorism
Affected domains	Linker between P2 domain and CBD	–	Linker between P2 domain and CBD	–	–	–	–	–
Zygosity	Hemi	Hemi	Hemi	Hemi	Hemi	Hemi	Het	Hemi
Method of detection	GS	ES	ES	Gene panel (215 ID and epilepsy genes)	ES	Gene panel (450 id genes)	Single exome	ES
Ancestry	Yemeni	Iranian	European	German	Indian	French	Turkish	European
Developmental delay	+	+	+	+	+	+	+	+
Intellectual disability	–	+	+	+	+	+	+	+

(Continues)

TABLE 1 | (Continued)

Subjects ID	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8
Learning disability	N/A	+	+	+	+	+	−	+
Autism	+	+	−	+	−	−	−	−
Epilepsy/ seizures/spasms	−	+	+	+	+	+	+	+
Language/ speech delay	+	+	+	+	+	+	+	+
ADHD	N/A	+	+	+	−	+	N/A	−
Behavioral problems	−	+	+	+	+	−	+	−
Anxiety disorder	N/A	N/A	+	−	−	+	−	−
Sleep disorder	N/A	+	−	−	N/A	−	+	−
Cranial anomalies	−	−	−	−	−	−	−	+
Dysmorphic features	−	+	−	−	+	NA	−	+
Hand/finger/ft/ toe anomalies	N/A	+ Mild tapering fingers. fetal pads in fingertips, a gap between 1st and 2nd toes, minor syndactyly between 2nd and 3rd toes.	+ Bilateral/3 toes syndactyly	−	+ Fetal pads in fingertips	−	−	−
Skeletal anomalies	N/A	−	−	−	−	−	−	+
Movement disorder	N/A	+	−	+	−	−	−	+
Hypotonia	N/A	−	−	+	+	−	−	−

(Continues)

TABLE 1 | (Continued)

Subjects ID	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8
Impaired motor skills (abnormal gait, ataxia)	N/A	N/A	−	+	+	−	−	+
Brain MRI	Normal	Normal	Normal	N/A	Normal	N/A	Normal	N/A
EEG	N/A	Abnormal continuous spike-and-slow-waves	N/A	Normal	Abnormal	N/A	Abnormal	+
Other	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Subjects ID	Subject 9	Subject 10	Subject 11	Subject 12	Subject 13	Subject 14	Subject 15
Nucleotide change NM_014927.5	c.298C>T	c.128A>G	c.228+1G>A	c.1090A>G	c.1235T>A	c.1235T>A	c.681+1G>A
Exon/intron	Exon 3	Exon 2	Intron 2	Exon 10	Exon 11	Exon 11	Intron 6
Sex	M	M	M	M	F	F	F
Current age	33 years	22 years	16 years	2 years	12 years	12 years	16 years
Effect on protein NP_055742.2	p.Gln100Ter	p.Gln43Arg	Splicing variant	p.Arg364Gly	p.Leu412Ter	p.Leu412Ter	Splicing variant
Genomic position HG 19	g.21450799C>T	g.21444678A>G	g.21444779G>A	g.21545117A>G	g.21550117T>A	g.21550117T>A	g. 21508697G>A
ACMG classification	PVS1, PS4, PM2, PM3, PP1, PP3, PP4 pathogenic	PS4, PM2, PM3, PP1, PP2, PP3, PP4 pathogenic	PVS1, PS4, PM2, PM3, PP1, PP3, PP4 pathogenic	PS4, PM2, PM3, PP1, PP2, PP3, PP4 pathogenic	PVS1, PS2, PS4, PM2, PP3 pathogenic	PVS1, PS2, PS4, PM2, PP3 pathogenic	PVS1, PS4, PM2, PP3, pathogenic
MAF (gnomAD)	0	0	0	0	0	0	0
M-CAP SCORE	N/A	Likely Benign	N/A	Possibly Pathogenic	N/A	N/A	N/A
CADD score	35	22.3	35	35	35	35	33

(Continues)

TABLE 1 | (Continued)

Subjects ID	Subject 9	Subject 10	Subject 11	Subject 12	Subject 13	Subject 14	Subject 15
Inheritance	Maternal; two older sisters with mild language delay, and one of them tested carried a heterozygous variant, asymptomatic mother carries the mosaic variant	Maternal	Maternal	Maternal; mother showed delayed development of motor function and speech	*de novo*	*de novo*	Not tested
Affected domains	–	SAM	–	DUF1170	–	–	–
Zygosity	Hemi	Hemi	Hemi	Hemi	Het	Het	Het
Method of detection	ES	Gene panel (450 id genes)	Gene panel (450 id genes)	ES	Gene panel (170 epilepsy genes)	Gene panel (170 epilepsy genes)	Gene panel (170 epilepsy genes)
Ancestry	European	Algerian	French	Romanian	French	French	French
Developmental delay	+	+	+	+	+	–	+
Intellectual disability	+	+	+	+	–	–	+
Learning disability	+	+	+	N/A	+	+	+
Autism	+	+	+	N/A	–	–	–
Epilepsy/seizures/spasms	+	+	–	–	+	+	+
Language/speech delay	+	+	+	+	–	–	+
ADHD	+	+	+	N/A	+	–	+
Behavioral problems	+	+	+	N/A	–	–	–
Anxiety disorder	+	N/A	+	N/A	–	–	+
Sleep disorder	+	+	–	–	–	+	+
Cranial anomalies	+	+	–	+ Plagiocephaly	–	–	–
Dysmorphic features	+	+	+	–	–	–	–

(Continues)

TABLE 1 | (Continued)

Subjects ID	Subject 9	Subject 10	Subject 11	Subject 12	Subject 13	Subject 14	Subject 15
Hand/finger/ft/toe anomalies	−	−	−	−	− (No major deformities but not specifically checked for minor)	− (No major deformities but not specifically checked for minor)	− (No major deformities but not specifically checked for minor)
Skeletal anomalies	−	N/A	−	−	−	−	Genu valgum
Movement disorder	+	−	−	−	−	−	−
Hypotonia	+	−	−	+	−	−	−
Impaired motor skills (abnormal gait, ataxia)	+	+ Spasticity of the ankles Walking on tiptoes Brisk tendon reflexes Balance disorder (abnormal gait)	−	−	−	−	−
Brain MRI	N/A	Abnormal moderate global cerebellar atrophy, brainstem hypoplasia, moderate bilateral hippocampal atrophy (19 yo)	Normal	Normal	Normal	Normal	Normal
EEG	Abnormal	Abnormal	Normal	Abnormal	Abnormal	Abnormal	Normal (at last follow-up but abnormal in the past)
Other	N/A	Generalized tonico-clonic seizures, absences	N/A	Soft connective tissue	N/A	N/A	History of DEE-SWAS

Note: 'N/A' denotes not available, while (−) represents absence of the corresponding phenotype. 'hemi' denotes hemizygous, while 'het' for heterozygous.

with autism, learning difficulties, and speech/language delay. The boy was born in Qatar to a 38-year-old Yemeni father and a 34-year-old Yemeni mother, delivered through a normal vaginal delivery following an uneventful pregnancy. Throughout the pregnancy, there were no vaccinations, medications taken, or prenatal complications. He had a birth weight of 3 kg, and there were no post-natal complications. He was breastfed for 12 weeks and then transitioned to bottle-feeding until the age of 2.5 years.

At home, the primary language spoken is Arabic, and the boy reached typical developmental milestones, such as babbling and saying "baba" and "mama" on time. He achieved independent sitting at 6 months and independent walking at 12 months. However, there were delays in his language and speech skills. While he began speaking single words like "baba" and "mama" at 12 months, he struggled with the formation of two-word sentences and complex sentence formulation. At 12 months of age, the boy experienced a temporary loss of acquired language skills, but there were no behavioral problems observed at home or outside, and he did not lose any other previously acquired skills. Additionally, at 12 months, the boy was hospitalized for Kawasaki disease but was later discharged. He also has an allergy to oranges. The autism diagnosis was made using the ADI-R by a psychologist in Qatar, although other tests like metabolic screening, EEG (electroencephalogram), fragile X-syndrome, tuberous sclerosis, and Rett syndrome tests have not been conducted, although CT/MRI scans yielded normal results. Currently 14 years old, he is right-handed and resides in Qatar.

2.1.2 | Subject 2 (c.1734G>A, P.Trp578Ter)

This subject is a 7-year-old Iranian boy from a consanguineous marriage (first cousin) displaying clinical features, including intractable seizure, ID, motor delay, language impairment, and autism spectrum disorder (ASD). He was born full-term via the cesarean section after a normal pregnancy, with a head circumference of 33 cm and a birth weight of 3200 g. While his neonatal and perinatal periods were uneventful, a sleep disorder emerged at the age of 4 months, which improved but later regressed as he grew older. There was no history of NDD in the family. During the first year, his motor and cognitive development were within normal range. However, in the second year, mild DD became apparent and worsened in the next year. At 27 months old, he experienced his first seizure, which was followed by recurrent episodes of focal seizures. An EEG showed continuous spike-and-slow-waves. Currently, he has a normal height, weight, and skeletal appearance. Various medications, including clobazam, acetazolamide, racosamide, and sodium valproate, were prescribed, but none have provided significant improvement. His seizure remained uncontrolled, with occasional episodes lasting less than a minute. Additionally, he was diagnosed with ASD by a pediatric psychiatrist based on the Autism Diagnostic Observation Schedule (ADOS) and attention deficit hyperactivity disorder (ADHD). Risperidone was prescribed for ADHD; however, it was discontinued promptly due to complications and worsening of seizures. He possesses prominent eyes with a tendency towards hypertelorism, alongside low-set and slanted ears, accompanied by a mild micrognathia. His hands exhibit gently tapering fingers, with a predisposition toward pointed fingertips, coupled with an interruption in the transverse distal palmar flexion crease (PFC), as well as a short vertical PFC. In his feet, there is a noticeable gap between the first and second toes, as well as slight syndactyly between the second and third toes. ES identified a pathogenic nonsense hemizygous variant, c.1734G>A p.Trp578Ter in *CNKSR2* (NM_014927.4). His mother had a homozygous wild type allele, suggesting the possibility of a de novo occurrence. The population frequency of this variant is null in the gnomAD and Iranome databases. In silico analysis predicted the detected nonsense variant to be deleterious and damaging, further supported by its high combined annotation dependent depletion (CADD) score of 37, which designates it among the top less than 0.1% of most deleterious variants.

2.1.3 | Subject 3 (Maternal C.2300C>T, p.Ser767Phe)

This patient is a 25-year-old Caucasian male with a familial history of ID with epilepsy, as seen in three maternal uncles. He was born full term via cesarean section, and his neonatal period was unremarkable. He began walking at 13 months, but speech delay was observed. At 8 years old, he was diagnosed with generalized seizures, which has been well controlled with valproate sodium. He was also diagnosed with ADHD and anxiety. His learning difficulties necessitated special education, and mild ID was noted. At 8 years old, he weighed 28 kg (mean) with a height of 128.5 cm (mean) and a head circumference of 53 cm (mean). No dysmorphic features were noted during physical examination except bilateral 2/3 toes syndactyly. Other completed tests, including *FMR1* methylation and array CGH, returned normal results. The brain MRI was normal. By employing singlet exome sequencing (ES), the missense variant c.2300C>T, p.Ser767Phe was identified in the proband III-1, with its presence also observed in his mother II-2 and affected uncle II-6 using Sanger sequencing, The pedigree indicates that this X-linked *CNKSR2* mutation has affected at least four consecutive generations (Figure 2B).

2.1.4 | Subject 4 (De Novo c.2026_2027delAG, p.Arg676Asp*fs**2)

A 12-year-old German male presents with an NDD. He presents with DD, autism, focal epilepsy, ADHD, motor and speech delay, and ID. Notably, during his early years, his morphological features resembled those of a boy with fragile X-syndrome. The patient was born prematurely at 36 weeks as the second twin, with a birth weight below the 10th percentile and a head circumference of 32 cm. During the neonatal period, he experienced severe myoclonus during sleep, but EEG results appeared normal, with myoclonus persisting until 8 months of age. In the first year, recurring bronchitis and pneumonia added to his medical challenges. At the age of 2 years, his mother sought medical consultation due to general DD, at which point neurology findings indicated signs of ataxia. Around the age of 5 years, he began experiencing epileptic seizures, mostly during sleep and frequently as status epilepticus. The patient received various therapies, including sultiam, etosuccimid, and valproat. Levetiracetam proved most effective in controlling seizures, although it led to severe behavior problems. For the last 2 years, he has been free from seizures, managing well with a combination of 12.5-mg

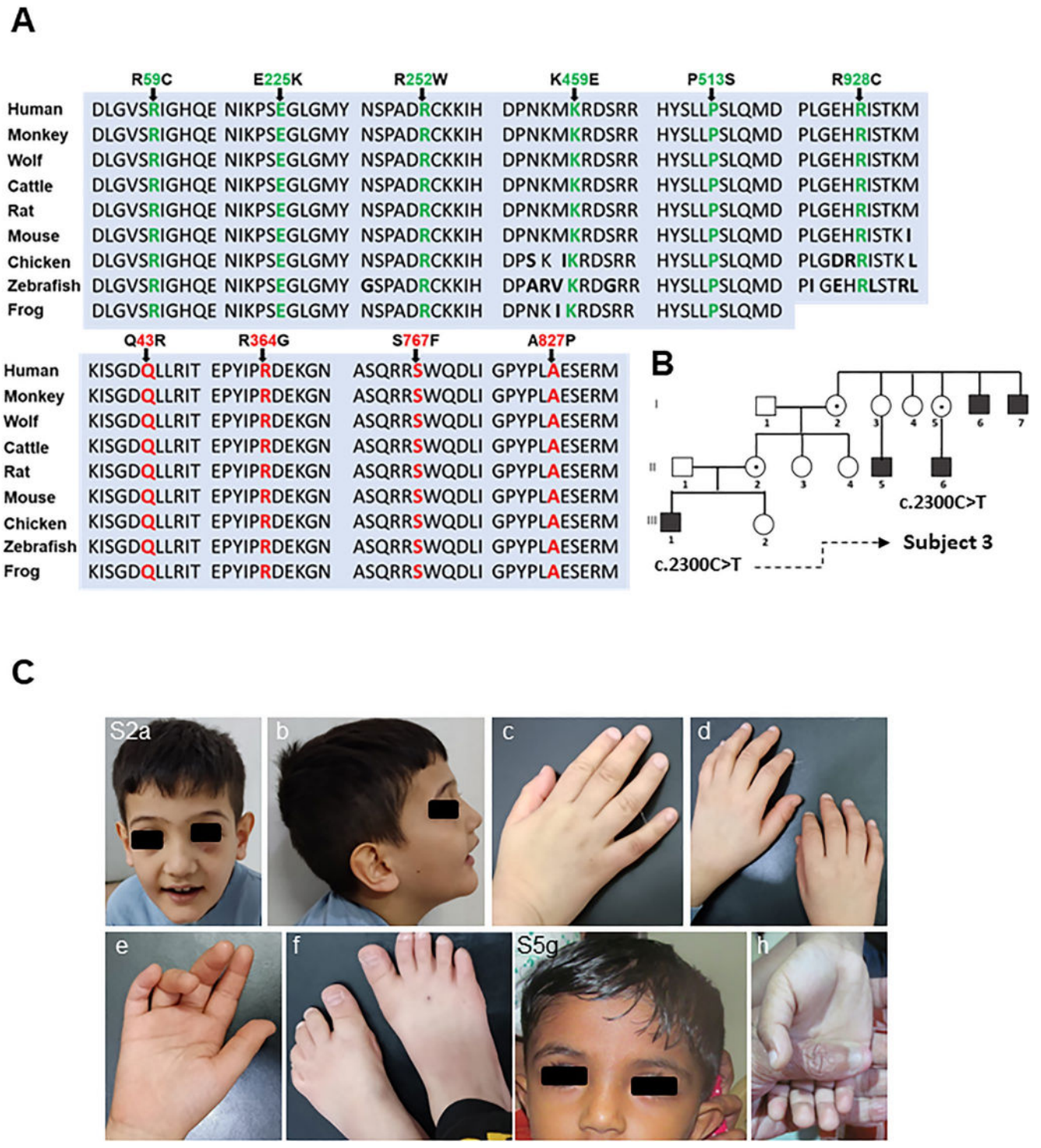

FIGURE 2 | (A) Conservation of ten crucial amino acids in CNKSR2 across nine vertebrate species. The CNKSR2 sequence alignment revealed that four amino acids (Q43, R364, S767, and A827) in our study and six amino acids (R59, E225, R252, K459, P513, and R928) from the reported papers were fully conserved among all nine vertebrate species, except R928, which is unavailable in frogs. The amino acids impacted by missense mutations in our patients were marked in bold red, while those in the reported papers were marked in bold green. (B) Pedigree of Subject 3, illustrating the *CNKSR2* mutation transmitted through a minimum of three successive generations. The mother of six offspring in generation I, although not depicted, is an obligatory carrier. (C) Phenotypic presentation of two pediatric cases with *CNKSR2* mutations: Subject 2 (S2), a 8-year-old boy, exhibiting features (a)–(f), and Subject 5 (S5), a 3.5-year-old boy, showing features (g) and (h). (a) big eyes with a tendency to hypertelorism (b) low-set and slanted ears accompanied by mild micrognathia (c), (d) mild tapering fingers (e) a tendency to pointed fingertips, disruption of the transverse distal palmar flexion crease (PFC), and a short vertical PFC (f) a gap between the 1st and 2nd toes and minor syndactyly between 2nd and 3rd toes (g) longer eyelashes, slanted eyes, lateral eversion of eyelids, and low-set ears (h) the presence of pointed fingertips.

brivaracetam, ospolot, and etosuccimid. Methylphenidate was used to address the ADHD symptoms.

At 8.8 years of age, the KABC-II NU scales were conducted to assess the patient's cognitive abilities. The KABC-II NU scales encompass five domains: Simultaneous (Gv), sequential (Gsm), planning (Gf), learning (Glr), and knowledge (Gc). The results showed scores of FCI 53, sequential 45, simultaneous 40, planning 40, long-term storage 74, and knowledge 74. Overall, the child demonstrates strengths in sequential processing, learning, and knowledge domains, scoring above the average range in these areas. The child's performance in simultaneous processing and planning domains falls within the average range compared to same-aged peers. A gene panel targeting 215 genes associated with ID and epilepsy identified a frameshift variant involving the deletion of 2 base pairs within the open reading frame, denoted as c.2026_2027delAG, p.Arg676Aspfs*2. Notably, this variant was absent in the Sanger sequencing of the mother's DNA of this boy, indicating that it likely arose as a de novo variant.

2.1.5 | Subject 5 (c.2041C>T, p.Gln681Ter)

This subject is a 3-year-old Asian (Indian) male with a medical history of ID, global DD, epilepsy, and mild dysmorphic features. He was born full term via lower segment cesarean section, weighing 3.3 kg (97th centile). Notably, he displayed hypotonia and did not achieve head control even by 6 months of age. Independent sitting was observed at 9 months, while walking began at 18 months. However, he had no effective speech at 3 years of age, prompting a speech and language assessment. His Receptive Expressive Emergent Language Scale (REELS) indicated delayed receptive language age (22–24 months) and expressive language age (8 to 9 months), at a chronological age of 3 years. On examination, he exhibited hypotonia and brisk reflexes, which led to the decision to perform a nerve conduction study (NCS) and a magnetic resonance imaging (MRI) of the brain. Both the NCS and MRI results were normal. A physical examination revealed a wide-based ataxic gait. He was diagnosed with seizures at 3 years of age, when he presented with recurrent episodes of motor arrest accompanied by head drops lasting for about 5 s. A prolonged 8-h video EEG showed multifocal epileptiform abnormalities, predominantly from the left parietal and temporal regions. He was diagnosed with focal clonic seizures, effectively controlled with sodium valproate and levetiracetam.

At 3 years of age he weighed 10.7 kg (< 3rd centile) with a height of 88 cm (< 3rd centile) and head circumference of 49 cm (15th–50th centile). He displayed several dysmorphic features, notably elongated eyelashes, slanted eyes, lateral eversion of the eyelid, and low-set ears. The presence of these distinct traits, couples with fetal pads of fingertips, prompted a clinical consideration of Kabuki syndrome. During follow-up at 3.5 years of age, he showed progress in speech, speaking in monosyllables after intensive speech and language intervention. Neurobehavioral assessment s did not reveal any disorders, and extensive metabolic workup, ultrasound imaging of the abdomen, and Brainstem Evoked Response Audiometry (BERA) all returned normal results.

2.1.6 | Subject 6 (c.2230delG, p.Glu744Serfs*6)

The patient, born in 1964, sought genetic counseling following a request from his niece (daughter of his sister). He presented with psychomotor delay and moderate intellectual deficiency. He can construct sentences and respond appropriately to simple questions, he struggles with reading, writing, and counting. Currently employed in a sheltered workshop, he demonstrates the ability to navigate to his office independently. Notably, he had no history of epilepsy or movement disorders and is described as quiet and friendly. He has no dysmorphic features. He has a brother affected with the same condition, experiencing seizures since childhood and exhibiting behavior problems such as anxiety and episodes of hallucinations. However, the brother was not assessed during the consultation. Testing for FMR1 expansion and array-CGH were negative. A gene panel analyzing 450 genes for ID revealed a frameshift variant of *CNKSR2* (c.2230delG), which was confirmed by Sanger sequencing. The variant was inherited from the patient's mother.

2.1.7 | Subject 7 (De Novo C.2336C>G, P.Ser779Ter)

This patient is a 6-year-old female with focal epilepsy and mild DD. She is the third child of consanguineous Turkish parents. Her pregnancy was uneventful, and she was born at 38 weeks gestation with a birthweight of 3360 g (52nd percentile), body length of 51 cm (53rd percentile) and head circumference of 36 cm (86th percentile). Her APGAR scores at 5 and 10 min were 10/10, and arterial pH was 7.30. Early development was considered as normal, achieving walking at 14 months and forming 2-word sentences at 20 months.

Seizures started at the end of her third year of life, initially presenting as mainly generalized episodes associated with acute infections, both febrile and afebrile. No treatment was initiated at this stage. In the early part of her fourth year, she experienced an episode of reduced reactivity while standing, accompanied by myoclonus of both arms and repetitive hand movement. This was interpreted as dyscognitive seizure, and focal epilepsy was diagnosed. Sleep-EEG revealed two distinct foci, one in the left frontal and the other in the right frontal. Cerebral MRI from an outside hospital showed no significant anomalies, although some concerns about image quality remained. Treatment with levetiracetam was initiated, and after one additional seizure at a dose of 40 mg/kg/day, the dosage was increased to 60 mg/kg/day, resulting in seizure freedom.

Recurring clinical examinations showed neither focal neurological deficit nor dysmorphic features. During the latest follow-up at 4 years and 9 months of age, she weighed 18.5 kg (55th percentile), with a body length of 112.5 cm (81st percentile), and a head circumference of 51 cm (34th percentile). A structured developmental assessment by occupational therapy (ET 6-6R) indicated age-appropriate results for gross motor function, but below-average results for fine motor function and cognition. A reduced attention span was also noted, and a formal psychometric examination is planned for further evaluation. She recently underwent formal neuropsychological testing, confirming language, and speech delay. She is currently receiving speech therapy and exhibits mild ID at the age of

6. Initially, her parents observed intermittent nighttime hypoxemia, likely due to nocturnal seizures during that period. Presently, she is displaying sleep-activated epileptiform potentials, reminiscent of developing continuous spike–wave in slow-wave sleep (CSWS), although not in a typical or complete manner at the moment.

2.1.8 | Subject 8 (Maternal C.1295dup, P.Thr433Asnfs*15)

Patient 8 is an 18-year-old male with ID, characterized by marked impairment of expressive language and a history of focal seizures. He is the first child of healthy European parents. His early psychomotor development was severely delayed, with focal seizures developing within the first years of life. However, his developmental progress was limited, especially in expressive language, which remained severely delayed, with only individual syllables articulated at the age of 4 years. In terms of physical examination, his body measurements fell within the normal range, and apart from intermittently pointed feet and a tendency bear weight on the medial part of his foot while walking, no other unremarkable findings were noted. At the age of 5 years and 6 months, he began speaking single words. His epileptic medication was discontinued at 14 years of age, and he has remained seizure-free since then. At 16 years of age, he attended a school for mentally handicapped children and showed some improvement in his ability to formulate simple sentences with good speech production. However, due to poor articulated speech, his communication was occasionally challenging to understand. A slightly elevated muscle tone was observed in his lower extremities, and a thoracolumbar scoliosis was documented. No dysmorphic features were noted except for hypertelorism.

Genetic tests, including chromosome analysis, subtelomer screening, and array CGH, yielded negative results. However, ES revealed a hemizygous frameshift variant in *CNKSR2* (c.1295dup, p.Thr433Asnfs*15) which was inherited from his mother, who showed mild symptoms.

2.1.9 | Subject 9 (Maternal C.298C>T, P.Gln100Ter)

This subject is a 30-year-old Caucasian male with a history of ID, autism, bipolar disorder with rapid cycling, petit mal epilepsy, motor apraxia, and muscular hypotonia. He was born at term by vaginal delivery to healthy parents. At the age of 8 months, he was noted to have muscular hypotonia; he made first steps at 19 months, spoke first words at 15 months, and was able to speak only few single words by the age of 3 years. He developed seizures from the age of 4 years onwards. His cognitive development remained delayed with poor language expression, slowed speech response, and poor social communication skills. He subsequently developed an ADHD and episodes of psychomotor agitation, impulsivity and insomnia, as well as periods of low mood and reclusiveness. At age 20 years, he was still unable to properly control his bladder and bowels. He attended a school for mentally handicapped children and was institutionalized with neuropsychiatric deterioration until now. At 30 years of age, he had hypotonic facies and a high forehead. Dysmorphic features included bushy low-set eyebrows, a short philtrum, and an upturned nose. Furthermore, he had retrognathia, prominent teeth, an overbite, as well as woolly, curly hair. Chromosome analysis and oligoarray/SNP analysis yielded negative results. ES revealed a hemizygous nonsense variant in *CNKSR2* (c.298C>T, p.Gln100Ter). His two older sisters showed mild language delay in early childhood but were able to attend regular school. They never suffered from seizures, although presenting with similar EEG abnormalities. Sanger sequencing of one of the two sisters with mild language delay confirmed her as a heterozygous carrier of the variant. Notably, the variant was observed with reduced allele frequency in a mosaic state (about 15% in blood-derived DNA) in his mother.

2.1.10 | Subject 10 (Maternal C.128A>G, P.Gln43Arg)

This male patient was first evaluated at the age of 2 years 5 months due to syndromic psychomotor retardation of unknown etiology. He is third child in a sibling group of three children, born to unrelated healthy parents. He was born at

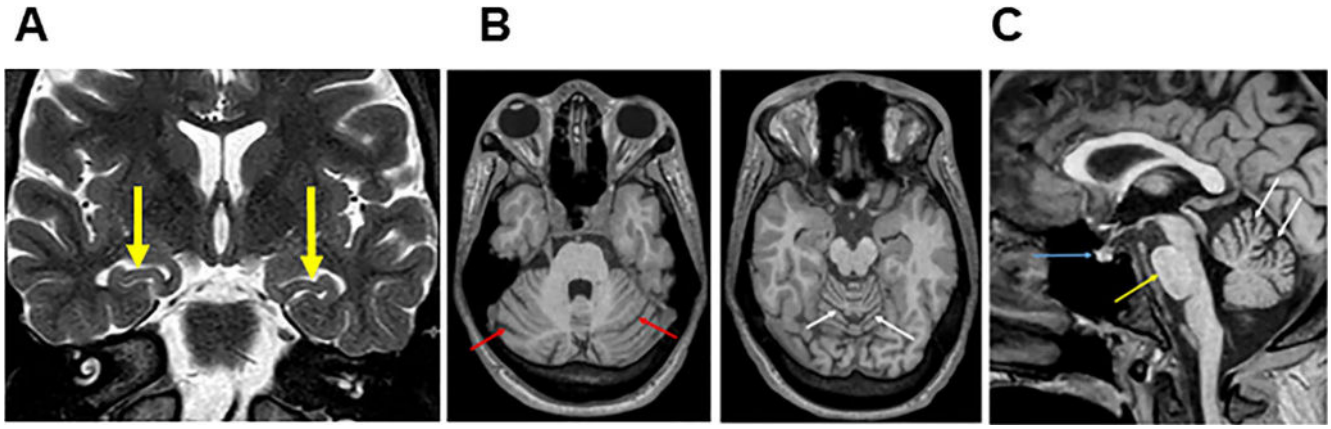

FIGURE 3 | (A) Coronal T2-weighted MRI showing significant volume reduction in both hippocampi, without any associated signal or morphological anomaly. (B) Axial T1-weighted MRI showing enlargement of hemispheric sulci (red arrows) and vermian sulci (white arrows), consistent with moderate diffuse cerebellar atrophy. (C) Sagittal T1-weighted MRI demonstrating moderate atrophy/hypoplasia of the cerebellar vermis (white arrows), brainstem (yellow arrow), and pituitary gland (blue arrow).

41 weeks of gestation after a normal pregnancy, with a birth weight of 3210 g, body height of 49 cm, and head circumference of 33 cm. His Apgar scores were 10 at 1 and 5 min. At 2 years 5 months, he was referred for evaluation due to delayed walking at 19 months and lack of acquisition of daytime and nighttime cleanliness. He also exhibited speech delay, only saying 2 words: "daddy" and "mummy." Dysmorphic features observed include hypertelorism, dystopia of the internal canthus of the eyes, flat and wide nasal root, low set hair on the forehead and the neck, short philtrum, drooping corners of the mouth, asymmetrical ears, small and round left ear, large hands with long and thin fingers. He displayed slowness of movement, staring, and kept his mouth open. Sleep disturbance was evident, with waking up around 3 am and going back to sleep around 11 am. Physical examination showed normal tendon reflexes, spasticity of the ankles, walking on tiptoes. He also had balance disorder with instability while standing and walking, and a widened sustentation polygon. Additionally, he experienced episodes of unmotivated laughter and comprehension impairment.

At 9 years 3 months, he had not acquired language and remained unable to achieve daytime and nighttime cleanliness. At 11 years 10 months, early puberty was observed, but no ataxia was noted. The patellar and Achilles tendon reflexes were slightly "sharp." He had a weight of 40.5 kg, height of 163 cm, and head circumference of 54.5 cm, with a moderately short uvula. A frontal spine x-ray showed scoliosis with Cobb's angle of about 10°. He achieved continence at the age of 13 years. Additionally, at 14 years 7 months, one probable brief nocturnal seizure was reported. He became more alert and attentive, and his speech improved slightly, with about 20 words. He could understand simple commands, pours himself a drink, and eat on his own. However, he primarily expressed himself by gestures. He experienced episodes of diurnal and nocturnal motor agitation and hypersalivation. By 19 years old, he was diagnosed with ID, ASD, epilepsy. Since the age of 14, he has been experiencing 1 to 2 seizures per year. At 17, absences began, followed by an increasing frequency of generalized tonic–clonic (GTC) seizures. Treatment with sodium valproate was not very effective, and zonegran (zonisamide) was added with good efficacy. Cardiac ultrasound revealed moderate stenosis of the left pulmonary artery and its branches. A negative metabolic work-up included assessments of urinary oligosaccharides and mucopolysaccharides, plasma acylcarnitine, chromatography of urinary organic acids, urinary amino acids and plasma amino acids, biotinidase activity, cholesterol metabolites, creatine deficiency, and standard biological tests.

Pulmonary edema assessment (PEA) results were normal, while Visual Evoked Potentials (VEPs) showed pathological findings. The electroretinogram (ERG) was normal. Polysomnographic recording indicated an obstructive or high resistance upper airway syndrome, short sleep cycle, decreased deep slow wave sleep time, and early arousal. Daytime melatonin levels were elevated, and beta-blocker treatment was administered. EEG findings showed long bursts of theta activity at 6 Hz over the anterior regions, along with frequent diffuse irregular spike discharges, especially during sleepiness and wakefulness, lasting 1–3 s. At the age of 11 years, the brain MRI revealed punctate hypersignals in the bifrontal subcortical white matter, along with discreet widening of the cerebellar vermis sulci. The EEG at 19 years indicated microvoltage brain activity without epileptic abnormality. The subsequent brain MRI at 19 years showed moderate bilateral hippocampal atrophy (Figure 3A), moderate global cerebellar atrophy (Figure 3B), along with atrophy or hypoplasia of the cerebellar vermis, brainstem, and pituitary gland (Figure 3C). His constitutional blood karyotype was normal, and tests for 17p FISH (fluorescence in situ hybridization), FRAXA (fragile X syndrome-A), and Angelman syndrome yielded negative results. Investigation for CDG syndrome and urinary succinylpurine test also came back negative. Array CGH array and MECP2 gene sequencing study showed normal results. A panel of 450 ID genes revealed a hemizygous variant, c.128A>G, p.Gln43Arg in *CNKSR2* in Subject 10. This variant was inherited from his mother, who is regarded to be in good health.

2.1.11 | Subject 11 (C.228+1G>A)

This boy, the only child of healthy nonrelated parents, showed intrauterine growth restriction during the 3rd trimester of pregnancy, leading to a cesarean section performed at 36 weeks gestation. At birth, his weight was 2 kg, his length measured 45 cm, and his head circumference was 31 cm. His APGAR score was 10/10. Hypotonia and global DD were noted from an early age. He started holding his head at 4 months, sitting at 1 year, and walking at 19 months. Speech delay and ID were noted as well as stereotypic movements. He attended a school for children with special needs and was treated by risperidone. Neither regression nor epilepsy were noted. Brain MRI, EEG, hearing test, and ophthalmological examination were reported to be normal, as were standard karyotype, chromosomal microarray, *FMR1* and *ARX* molecular analyses, and urine screening for inborn errors of creatine metabolism. Physical examination revealed downslanting palpebral fissures, but no other obvious dysmorphic features were observed.

At the age of 12 years and 4 months, his head circumference was 52.7 cm (−0.5SD), his height was 151 cm (+0.5SD), and his weight was 36.8 kg (0SD). The molecular analysis of a panel of genes associated with NDDs revealed a likely pathogenic splice variant c.228+1G>A in *CNKSR2*, inherited from his asymptomatic mother.

2.1.12 | Subject 12 (Maternal C.1090A>G, P.Arg364Gly)

Patient 12 is a 2-year and 1-month old Caucasian male. He is the first child of Romanian parents. His mother showed a delayed development of motor function and speech. The patient was born in Romania in the 34th week of gestation with a birth weight of 2090 g (27th centile, −0.6 z), length of 45 cm (34th centile, −0.4 z) and head circumference of 30 cm (10th centile, −1.3 z). The Apgar score was 7. He received CPAP ventilation for the first 2 days of life due to respiratory distress and underwent antibiotic therapy due to suspected bacterial infection. At the age of 3 months, muscle hypotonia was noticed, and physiotherapy was initiated at the age of 8 months. The family relocated from Romania to Germany when he was 16 months old. He first visited this clinic at 20 months of age. At that time, his body length

was 82.5 cm (69th centile, +0.5 z), body weight was 11 kg (49th 0 z), and head circumference was 46.5 cm (9th centile, −1.3 z). He showed a plagiocephaly and muscle hypotonia with soft connective tissue. Deep tendon reflexes were absent. While he was able to sit independently for only a short time, he showed thoracal kyphosis and was not able to crawl or stand. His speech was limited to a few single words, and word understanding was not age-appropriate. The parents reported that 5q-associated spinal muscle atrophy has been genetically excluded, although there was no written report from Romania. Laboratory examination at 16 months showed normal values for creatine kinase and acid alpha-glucosidase, along with extended metabolic analyses in blood and urine. *SMN1* analysis was normal, showing two copies of exon 7 and exon 8. Motor conduction velocity was also normal. At 24 moths, cerebral MRI was normal, especially without any signs of perinatal residuals. EEG showed bilateral parietal sharp slow waves that activated during sleep, but no electrical status during sleep occurred, and there was no history of seizures. At the age of 26 months, he has started to come to the quadruped stand and can only say the words "mama" and "papa."

2.1.13 | Subject 13 (De Novo C.1235T>A, P.Leu412Ter)

Subject 13, a female, was delivered at 37 weeks of gestational age (GA) as part of an uneventful dichorionic diamniotic twin pregnancy involving two healthy and non-consanguineous parents. The neonate was appropriate for GA (AGA), weighing 2625 g (25th percentile), and had an uncomplicated perinatal history. The detailed medical history of her twin sister will be provided below. There was a positive family history of epilepsy in the sister and the paternal grandmother on the subject's mother's side. During the initial years of life, her growth and development progressed within the normal range. She began speaking her first words before reaching 12 months of age and started walking independently at 16 months. However, at the age of 3 years and 2 months, she experienced her inaugural focal seizure. This seizure subsequently developed into drug-resistant, unclassifiable epilepsy characterized by GTC, focal motor, non-motor seizures, and focal-to-bilateral tonic–clonic seizures.

Multiple electroencephalograms conducted during her follow-up consistently displayed a normal background activity. Interictal bilateral asynchronous focal (primarily anterior) epileptic abnormalities, activated during sleep, were also observed. The specific epileptic foci varied in location between recordings, initially suggesting the potential for self-limited epilepsy. Additionally, she received a diagnosis of NDD, marked by prominent attention deficits, executive function challenges, and difficulties in learning, particularly in reading and writing. Her physical examination was otherwise normal. Despite an extensive etiological investigation, which included brain MRI, CSF/blood metabolic analyses, and molecular karyotyping, no conclusive results were obtained. However, an epilepsy gene panel analysis identified a de novo heterozygous variant (c.1235T>A, p.Leu412Ter) in *CNSKR2*, which was also identified in her twin sister.

During the most recent follow-up at the age of 11 years and 8 months, her epilepsy was effectively managed through a combination therapy involving valproate and levetiracetam. GTC seizures were absent, and infrequent short focal motor seizures occurred primarily during sleep. She was enrolled in a specialized education program within a regular middle school setting, where she received comprehensive in-class support from an educator. Additionally, she participated in speech therapy and neuro-feedback sessions four times a week to address attention and learning difficulties. Her cognitive abilities, assessed most recently, fell within the lower bounds of the normal range for her age, and her IQ could not be precisely determined.

2.1.14 | Subject 14 (De Novo C.1235T>A, P.Leu412Ter)

Subject 14, a female, is the twin sibling of Subject 13. The details of the pregnancy, family background, and medical history of the twin sister have been outlined previously. She was born AGA, with a birth weight of 2.390 g (15th percentile), and had an uneventful perinatal history. She had her first episode of febrile seizure during an episode of pyelonephritis when she was 3 months old. Subsequently, at three and a half years of age, a few months after the onset of epilepsy in her twin sister, she presented a second febrile seizure. Soon afterwards, her symptoms evolved into drug-resistant epilepsy, initially marked by GTC seizures, followed by focal motor and non-motor seizures.

Although her neurodevelopment was deemed typical, she faced challenges in learning, including dyslexia and dyscalculia, during her early schooling years. Additionally, an anxiety disorder emerged. She also experienced migraine headaches, which were managed with relief medications, and had sleep disturbances. Her physical examination was normal. EEG mirrored her sister's, displaying normal background activity and interictal abnormalities, primarily manifesting as independent spikes in the right or left anterior (mainly frontal) regions, with activation during sleep. The initial diagnostic investigation, identical to that of Subject 13, was normal. However, an epilepsy gene panel analysis confirmed the presence of a de novo heterozygous variant (c.1235T>A, p.Leu412Ter) in *CNSKR2*, consistent with her twin sister's condition.

As of the most recent follow-up, at the age of 11 years and 8 months, she had been seizure-free for over 2 years, maintained under levetiracetam monotherapy. She was successfully enrolled in a standard middle school education program and received one weekly session of speech therapy. Her cognitive abilities and IQ fell within the normal range during the last neuropsychological assessment, and her earlier reports of anxiety and sleep disorders were no longer present. However, she continued to experience frequent migraine headaches.

To our best of knowledge, this case marks the first documented report of dizygotic twin females carrying a de novo mutation in *CNKSR2*. Despite sharing a similar trajectory of epilepsy and EEG patterns, the developmental progression and outcomes for Subject 14 are significantly better than those of her sister Subject 13. This underscores the existing inquiries surrounding gene expression and phenotypic diversity in females.

2.1.15 | Subject 15 (C.681+1G>A)

Subject 15, a female, was born at 37 weeks of GA following an uncomplicated pregnancy involving two healthy,

non-consanguineous parents. Her Apgar score at birth was within the normal range. Her birth weight measured 2200 g (6th percentile), and her height measured 44.5 cm (6th percentile), indicating a symmetric small-for-gestational-age neonate. The family history was unremarkable. Her initial development was described as normal; she could walk and speak by 14 months of age. However, due to recurrent ear infections, she underwent tympanostomy procedures twice.

At the age of 2 years, she presented a single febrile seizure without any complications. A non-febrile GTC seizure occurred when she was three and a half years old. Following an abnormal EEG recording, she was prescribed an anti-seizure medication valproate. Over the next few months, changes in her behavior, including irritability, accompanied by speech regression followed by a global developmental stagnation, led to the diagnosis of developmental and epileptic encephalopathy linked to continuous spikes and waves during sleep (DEE-CSWS). This diagnosis was subsequently confirmed through a 24-h EEG monitoring conducted at our facility. While there was an initial improvement in her condition with the combined use of clobazam and valproate, she subsequently experienced further developmental stagnation. This was compounded by various seizure types, including GTC seizures, eyelid myoclonia with or without absences, and atonic seizures characterized by sudden head drops. Additionally, CSWS persisted. Extensive investigations, including brain MRI, glucose CSF/blood ratio assessment, molecular karyotyping, and screening for *GRIN2A* and *FMR-1* mutations, all yielded normal results. Despite attempts involving numerous antiseizure medications, corticosteroids, and a ketogenic diet, the patient only experienced partial or no improvement. Starting from the age of 12 years, a gradual improvement in both electroclinical parameters was observed. At the most recent follow-up, at 15 years of age, the patient displayed a global DD. She was capable of independent walking and exhibited some level of autonomy in basic daily activities. While oral communication was possible, it was subject to limitations, including echolalia. To manage her frequent awakenings, she was prescribed melatonin, and methylphenidate was used to address her attention deficit. Furthermore, signs of anxiety were noted. In the preceding 3 years, she had been free of GTC seizures while under monotherapy with ethosuximide. Nonetheless, infrequent episodes of eyelid myoclonia persisted, sometimes occurring in clusters. These episodes were responsive to oral administration of clobazam over several days. Prolonged EEG monitoring showed a normal background without any paroxysmal activity. To complete the etiological investigation, an epilepsy gene panel was employed. This analysis identified a heterozygous mutation in the *CNKSR2* gene, yielding a positive result. To date, the exact inheritance pattern remains undisclosed.

2.2 | Multiple Protein-Sequence Alignment of CNKSR2 With Its Orthologs

The four amino acids affected by missense mutations—Gln43, Arg364, Ser767, and Ala827—along with the six reported missense mutations—Arg59, Glu225, Arg252, Lys459, Pro513, and Arg928—show complete conservation in the protein sequence across nine vertebrate species, with the exception of Arg928, which is absent in frogs (Figure 2A). This highlights the detrimental impact of these substitutions and underscores their critical role in shared biological processes across species. With five nonsense (Gln100Ter, Leu412Ter, Trp578Ter, Gln681Ter, Ser779Ter), two splice-site (c.228+1G>A and c.681+1G>A), and three frameshift mutations (Thr433Asnfs*15, Arg676Aspfs*2, Glu744Serfs*6) found in our patients, CNKSR2 mutant proteins with any of these variants are likely non-functional, representing loss-of-function mutations.

2.3 | Molecular Analysis With Exome Sequencing and Gene Panels

By employing ES and panels of ID and epilepsy genes, we conducted an investigation involving 15 MRXSHG patients from 14 different families across various countries. This study revealed a spectrum of 14 distinct alterations within the *CNKSR2* gene. These alterations encompass three frameshift variants, five nonsense variants, four missense variants, and additionally, two splice site variants (as illustrated in Table 1 and Figure 1). The ACMG interpretation of these 14 variants consistently labeled them as pathogenic, underscoring the detrimental impact of these *CNKSR2* genetic alterations (Table 1).

Among these cases, 11 are males, and four are females. These cases were recruited from diverse countries across three continents, including Asia (Yemen and India), Europe (Germany, France, and Romania), Eurasia (Turkey and Iran), and Africa (Algeria). The ages ranged from 2 years to 57 years old. Detailed clinical presentations of each case can be found in the section of Human Subjects. Notably, DD was universally observed in all cases except for one of the female cases, while other common neurological features included language/speech delay (13/15), ID (12/15), learning disability (12/15), and seizure (12/15). A variety of seizure types, such as clonic, myoclonic, tonic–clonic, atonic, absence, focal, status epilepticus, and febrile seizures, were observed among the cases. EEG results were available for twelve cases, with three of them showing no abnormal findings.

Regarding psychobehavioral features, our cases exhibited autism (6/15), behavioral problems (8/15), anxiety disorder (5/15), and ADHD (9/15). Further clinical information for each of 15 cases can be found in Table 1 and in the section of "Human Subjects."

Using WES, we identified 14 distinct variants across 15 cases. These variants were subsequently validated in each case and their respective parents through Sanger sequencing, except for Subject 15. Among these variants, nine were maternally inherited, four were *de novo*, and inheritance pattern of one variant could not be determined. Among the maternally inherited variants, Subject 8's mother exhibited hypertelorism as a dysmorphic feature. Subject 9 inherited the hemizygous nonsense variant from his asymptomatic mother with the variant mosaicism. His two older sisters had mild language delay, and Sanger sequencing of one of them confirmed her as a heterozygous carrier of the variant. Additionally, the mother of Subject 12 displayed a delayed development of motor function and speech. The details of variant types, positions, their effect on the protein, and individual phenotypes can be found in Table 1.

3 | Discussion

We present 15 patients with *CNKSR2* mutations, including three from the MENA (Middle East and North Africa) region, where such mutations have not been previously reported. Genetic analyses identified 14 distinct *CNKSR2* variants, including five nonsense, three frameshift, two splice, and four missense variants, 13 of which are novel. The study also revealed new digital and brain phenotypes, such as pointed fingertips (fetal pads of fingertips), syndactyly, tapering fingers, and hippocampal atrophy. These novel clinical features, along with the 13 new variants, further expand the phenotypic and genotypic spectrum of MRXSHG associated with *CNKSR2* mutations.

Out of the 14 *CNKSR2* variants we have identified, the *de novo* frameshift variant c.2026_2027delAG, p.Arg676Aspfs*2 identified in a German Subject 4 has been previously documented in three different papers (Higa et al. 2021; Turner et al. 2019; Martin et al. 2021), likely referring to the same patient in two papers (Turner et al. 2019; Martin et al. 2021). Notably, the third paper provides a detailed description of the phenotype exhibited by a 8-year-old boy affected by the same de novo variant (Higa et al. 2021). Our Subject 4, a 12-year-old boy, was born prematurely and displayed signs of ataxia at 2 years old. His epilepsy started around age 5, and he was later diagnosed with autism and ADHD. However, his EEG results were normal. In contrast, the reported 8-year-old boy with the same variant was born full term and was diagnosed with seizures at the age of 2. His EEG at age 8 indicated the presence of ESES (electrical status epilepticus during sleep). Similar to Subject 4, he also received diagnoses of autism and ADHD (Higa et al. 2021).

EEG analysis of Subject 12 revealed bilateral parietal sharp slow waves activated during sleep. Despite this, there was no occurrence of electrical status during sleep, and the patient had no history of seizures at the age of 25 months.

Strikingly, the four amino acids impacted by missense mutations—Gln43, Arg364, Ser767, and Ala827—demonstrate complete conservation in protein sequence across nine vertebrate species, emphasizing the detrimental consequences of these substitutions. Likewise, protein alignment of the six amino acids affected by the reported missense mutations displayed comparable outcomes, accentuating their vital involvement in shared biological processes across these species (Figure 2A). This implies that CNKSR2 mutant proteins with any of these missense variants are likely non-functional, representing loss-of-function variants. This underlying mechanism was confirmed by human patients with deletions encompassing the entire *CNKSR2* (Aypar, Wirrell, and Hoppman 2015; Vaags et al. 2014) and conducting a literature review (Higa et al. 2021).

Out of the 11 male subjects in this study, six harbor protein-truncating alterations, encompassing three nonsense and three frameshift variants. Since 2011, the literature has documented a total of 25 truncating mutations, consisting of 12 nonsense and 13 frameshift mutations (Figure 1). Notably, the nonsense mutation, Gln209Ter (c.625C>T) (Kang et al. 2021) is conspicuously absent from the HGMD website due to an oversight. Out of 30 reported cases with truncating mutations, 21 are male and accompanied by detailed clinical data description. In these cases, DD, speech/language delay, and ID were common phenotypes. Seizures were also reported in all except two subjects, with age onset ranging from 6 months to 4 years. The most frequent behavioral psychiatric manifestations included ID, hyperactivity, autism, and ADHD, although some cases did not exhibit any behavioral or psychiatric problems at all. Most patients showed no abnormal findings in MR imaging, with only three cases separately reporting severe hydrocephalus, minor cortical atrophy and periventricular and subependymal heterotopia. Additionally, some cases presented with feeding difficulties, dyspraxia, hypotonia, and macrocephaly (Damiano et al. 2017; Vaags et al. 2014; Higa et al. 2021; Bonardi et al. 2020; Kang et al. 2021; Liu et al. 2022b). Consistent with previous reports, DD (6/6), speech/language delay (6/6), ID (6/6), and seizure (6/6) were shared among six male subjects with nonsense or frameshift mutations in our study (Subjects 2, 4, 5, 6, 8, 9). As for psychobehavioral conditions, our male subjects with truncating variants showed behavioral problems (4/6), ADHD (4/6), and anxiety disorder (2/6). Furthermore, hypotonia and sleep disorder were found in three and two subjects, respectively. Even though facial dysmorphism is a rare finding in MRXSHG, six cases among our 15 Subjects showed facial dysmorphisms. Subject 2 presents with prominent eyes and a mild hypertelorism, accompanied by low-set and slanted ears, along with mild micrognathia. In contrast, Subject 5 is characterized by long eyelashes, lateral eversion of the eyelids, slanted eyes, and fetal pads on the fingertips. Notably, no dysmorphic features were detected in Subject 8, apart from hypertelorism, which was also evident in his mother. Subject 9, at the age of 30, exhibits hypotonic facies and a notably high forehead. His distinctive attributes include bushy, low-set eyebrows, a shortened philtrum, and an upturned nose. Furthermore, retrognathia, prominent teeth, and an overbite are also noticeable features. For Subject 10, hypertelorism, dystopia of the internal canthi of the eyes, a flat and wide nasal root, low-set hair on the forehead and neck, short philtrum, drooping corners of the mouth, asymmetrical ears, and a small, round left ear are evident. Subject 11 exhibits downslanting palpebral fissures, with no other apparent dysmorphic features.

Moreover, fetal pads in fingers (pointed fingertips), typically seen in Kabuki syndrome, were observed in Subjects 2 and 5. Syndactyly was detected in Subjects 2 and 3, while Subject 2 exhibited additionally tapering fingers (Figure 2C). Subject 10 displayed moderate bilateral hippocampal atrophy (Figure 3). These novel digital and brain phenotypes had previously never been documented in MRXSHG in the literature before. Up until now, only clinodactyly and brachydactyly have been documented as digital phenotypes in a girl with a de novo heterozygous nonsense mutation c.2304G>A, p.Trp768Ter in *CNKSR2* (Polla et al. 2019). The three novel variants were identified in distinct individuals: one from Yemeni in Qatar (Subject 1), another from Iran (Subject 2), and a third from Algeria, as detailed in Table 1. Importantly, instances of *CNKSR2* variants have not previously been reported in the MENA (Middle East and North Africa) region rendering this discovery both novel and significant. This finding underscores the global occurrence of *CNKSR2* variants, irrespective of individuals' genetic backgrounds.

In certain instances, the female proband herself or female family members of male probands exhibited a comparatively milder phenotype. Common phenotypes among the four female probands in this study were epilepsy/seizures/spasms (4/4), DD (3/4), learning disability (3/4), and sleep disorder (3/4). However, ADHD (2/4), behavioral problems (1/4), anxiety disorder (1/4), and autism (0/4) were less common (Table 1). The mother, who carried the heterozygous variant and transmitted it to her son (Subject 8), displayed hypertelorism. Additionally, two older sisters of Subject 9 showed mild language delay in early childhood but were able to attend regular school and never suffered from seizures. One of the sisters was tested for p.Gln100Ter variant and found to be heterozygous. The variant c.298C>T, p.Gln100Ter was detected in a mosaic state, showing a diminished allele frequency (approximately 15%) in blood-derived DNA from the asymptomatic mother of Subject 9. Notably, a case of mosaicism in an asymptomatic mother with a low frequency (5/156 reads) has been documented (Bonardi et al. 2020). This mosaicism affected her son who presented with the hemizygous variant c.457_461delTATTC, p.Tyr153Serfs*5. This marks the second instance of mosaicism involving *CNKSR2*. The mother of Subject 12, carrying the heterozygous variant c.1090A>G, p.Arg364Gly, exhibits mild symptoms with delayed development of motor function and speech.

3.1 | Limitation

The limitations of our study stem from a relatively small sample size of 15 patients, potentially impacting the generalizability of our findings and reducing our statistical power. Furthermore, our research did not encompass functional experiments to validate the impact of *CNKSR2* mutations on protein function or signaling pathways.

4 | Conclusion

In this study, we clinically evaluated 15 NDD patients and molecularly diagnosed them with MRXSHG by identifying *CNKSR2* variants. Our analysis confirmed variant distribution across the *CNKSR2* gene, primarily clustering at the 3′ end. Notably, we report the first cases of MRXSHG patients in the MENA region, featuring three novel *CNKSR2* mutations.

Importantly, mutations Ser767Phe and Ala827Pro may lead to proteasomal degradation or reduced PSD size, contributing to the neurodevelopmental phenotype. Furthermore, these two amino acids, along with another two affected by four missense variants, exhibit complete conservation across nine vertebrate species, highlighting their crucial role in the gene's functionality. Our study revealed unique new digital and brain phenotype, including pointed fingertips, syndactyly, tapering fingers, and hippocampal atrophy. These novel clinical features in MRXSHG, combined with the identification of 13 novel *CNKSR2* variants, broaden both the phenotypic and genotypic spectra associated with MRXSHG caused by *CNKSR2* mutations.

5 | Materials and Methods

5.1 | Human Subjects

The study sample consisted of 15 patients diagnosed with NDDs, encompassing DD, ID, ASD, epilepsy, and ADHD, who originated from 14 different families. These patients were referred to the clinical genetics department by neurologists, psychiatrists, and geneticists for either ES or targeted gene sequencing. A team of multidisciplinary specialists in a collaborative clinic collectively assessed all individuals, thus confirming the presence of neurodevelopmental dysfunction. Subsequently, following written informed consent from the families, comprehensive clinical assessments were conducted before proceeding to genetic and genomic analysis.

5.2 | Exome Sequencing

ES was performed on the probands to detect pathogenic variants using a custom-designed NimbleGen chip capturing array. Approximately 60 Mb of the targeted region on consensus coding sequences enriched from fragmented genomic DNA of the probands was covered by around 758,086 probes designed for the human genome (Agilent SureSelectXT2 V6 exome). Subsequently, paired-end sequencing was conducted on the NovaSeq6000 platform with a read length of 250 bp, and 100X coverage, following the manufacturer's protocol (Illumina, San Diego, CA, USA).

The resulting Fastq files were trimmed and aligned with the human reference genome (GRCh37/hg19) using Burrows-Wheeler Aligner (BWA) software. Variant calling was performed by SAMTools and Genome Analysis Toolkit (GATK v3.7) on the BAM file (Genomes Project C et al. 2010; Li and Durbin 2010; Li et al. 2009; McKenna et al. 2010; Cingolani et al. 2012). ANNOVAR software was used to annotate and filter the variants against dbSNP138. All pathogenic variants reported in Human Gene Mutation Database (HGMD) were considered, as along with variants with minor allele frequency (MAF) less than 0.01%, which were filtered against gnomAD, ExAC, 1000Genome projects, dbSNP138, ESP6500, NHBL Exome Variant Server (EVS). Additionally, for Subject 2, variants were filtered against the Iranome database. All rare and novel coding and splicing variants were categorized based on being nonsynonymous, indel, and putative splice sites. Predictor tools for determining the candidate variants' pathogenicity included Polyphen2, SIFT, MutationTaster, and CADD software (Kircher et al. 2014; Schwarz et al. 2014; Adzhubei et al. 2010; Kumar, Henikoff, and Ng 2009; Salgado et al. 2016; Keshava Prasad et al. 2009). To confirm and segregate the resulting variants, Sanger sequencing was performed on the affected individuals, their mothers, and siblings when available.

5.3 | Gene Panel Sequencing (215 Genes for ID and Epilepsy)

Genomic DNA was isolated from EDTA-blood according to the manufacturers' instructions using the QIAamp DNA

Blood Maxi Kit on a QiaSymphony instrument (Qiagen, Hilden, Germany). DNA quantity and quality were assessed using QubituFluorometer and NanoDrop ND-8000 (Thermo Fisher Scientific, Dreieich, Germany). The coding and flanking intronic regions were enriched using in solution hybridization technology and were sequenced using the Illumina HiSeq/NovaSeq system. Adapter sequences were removed with Skewer and the sequences obtained, were aligned to the human reference genome (hg19) with the Burrows Wheeler aligner (BWA mem). Sequences that could not be clearly assigned to a genomic position were removed, as were sequence duplicates that were probably due to amplification (internal software). Sequence variants (single nucleotide exchanges and short insertions/deletions) were determined from the remaining high-quality sequences using CeGaT StrataCall. Copy number variations (CNV) were computed on uniquely mapping, non-duplicate, high quality reads using an internally developed method based on sequencing coverage depth. Briefly, we used reference samples to create a model of the expected coverage that represents wet-lab biases as well as inter-sample variation. CNV calling was performed by computing the sample's normalized coverage profile and its deviation from the expected coverage. Genomic regions are referred to as variants if they deviate significantly from the expected coverage. Only variants (SNVs/Small Indels) in the coding region and the flanking intronic regions (±8 bp) with a MAF < 1.5% are evaluated. Known disease-causing variants (according to HGMD) are evaluated in up to ±30 bp of flanking regions and up to 5% MAF. Minor allele frequencies are taken from public databases (e.g., gnomAD) and an in-house database. Resulting variants were annotated with population frequencies from gnomAD and an internal database, factoring in external databases (e.g., HGMD, ClinVar), and with transcript information from Ensembl, RefSeq, Gencode, and CCDS. All variants were manually assessed before inclusion in the final report.

5.4 | Gene Panel Sequencing (450 ID Genes)

DNA was extracted from peripheral blood samples using the Nucleospin blood kit (Macherey Nagel, Hoerdt, France). Subsequently, paired-end sequencing (2 × 75 bp) was performed on a NextSeq500 instrument (Illumina Inc., San Diego, CA, USA) after standard library preparation (SeqCap EZ, Roche, Pleasanton, CA, USA). This sequencing specifically targeted 450 genes that have previously been associated with NDDs. For variant analysis, we employed an in-house bioinformatics pipeline adhering to the GATK v3.5 best practice guidelines (https://software.broadinstitute.org/gatk/best-practices). Our focus was on identifying rare variants (with a MAF < 1%) including non-synonymous, splice site, and indel variants.

5.5 | Gene Panel (170 Epilepsy Genes)

Utilizing the Nucleospin blood kit (Macherey Nagel, Hoerdt, France), DNA was extracted from peripheral blood samples. The subsequent paired-end sequencing (2 × 75 bp) was performed on a NextSeq500 system (Illumina Inc., San Diego, CA, USA), following standard library preparation (SeqCap EZ, Roche, Pleasanton, CA, USA) targeting 170 genes previously implicated in monogenic epilepsies. For the variant analysis, we used an in-house bioinformatics pipeline aligned with the GATK v3.5 best practice guidelines (https://software.broadinstitute.org/gatk/best-practices) and analyzed rare variants, including non-synonymous, splice site, and indel variants, with a MAF < 1%.

Author Contributions

M.R.G. and S.T.F. conceived the study, analyzed the data, and drafted the initial manuscript including Table under the supervision of M.M. A.B.-M. and V.G. undertook patient recruitment, compiled clinical data, and generated Tables and Figures. L.G.S., G.L., N.C., K.P., P.E., I.B., B.C., H.L.S., I.K.-S., R.P., S.N., S. Syrbe, U.P., S. Spranger, K.G.-H., T.B.H., M.T.P., T.d.S.G., E.P., A.A., S.H.T., M.R., and G.C.K. performed genetic analyses, curated and analyzed data, and wrote clinical reports. H.S., R.M., S.A., M.-H.J., and L.C.L. contributed to manuscript editing and data analysis. Y.L. evaluated the patients' clinical features. M.M. conducted data analysis, composed the manuscript including clinical reports, and acquired the funding. H.-G.K. supervised A.B.-M. and V.G., acquired the funding, drafted, edited, finalized the manuscript. All authors reviewed and approved the final manuscript.

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Ethics Statement

The studies involving human participants received ethical approval from the Ethical Committee, deputy of research affairs of Shahid Beheshti University of Medical Sciences, Tehran, Iran with approval code: IR.SBMU.MSP.REC.1399.112. and the Institutional Review Board of Augusta University, Georgia, USA.

Consent

Written informed consent for the publication of images and clinical data included in this article was obtained from all study participants and/or their legal guardians.

Data Availability Statement

The variants dataset presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/clinvar/, RCV000785133, RCV000782047, RCV001008396, RCV001638189, RCV001728040, RCV001253675, RCV001682664, RCV001268320, RCV000760277.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.