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Original Article

Semaphorin-Plexin Signaling: From Axonal Guidance to a New X-Linked Intellectual Disability Syndrome



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ABSTRACT

Background: Semaphorins and plexins are ligands and cell surface receptors that regulate multiple neurodevelopmental processes such as axonal growth and guidance. *PLXNA3* is a plexin gene located on the X chromosome that encodes the most widely expressed plexin receptor in fetal brain, plexin-A3. Plexin-A3 knockout mice demonstrate its role in semaphorin signaling *in vivo*. The clinical manifestations of semaphorin/plexin neurodevelopmental disorders have been less widely explored. This study describes the neurological and neurodevelopmental phenotypes of boys with maternally inherited hemizygous *PLXNA3* variants.

Methods: Data-sharing through GeneDx and GeneMatcher allowed identification of individuals with autism or intellectual disabilities (autism/ID) and hemizygous *PLXNA3* variants in collaboration with their physicians and genetic counselors, who completed questionnaires about their patients. *In silico* analyses predicted pathogenicity for each *PLXNA3* variant.

Results: We assessed 14 boys (mean age, 10.7 [range 2 to 25] years) with maternally inherited hemizygous *PLXNA3* variants and autism/ID ranging from mild to severe. Other findings included fine motor dyspraxia (92%), attention-deficit/hyperactivity traits, and aggressive behaviors (63%). Six patients (43%) had seizures. Thirteen boys (93%) with *PLXNA3* variants showed novel or very low allele frequencies and probable damaging/disease-causing pathogenicity in one or more predictors. We found a genotype-phenotype correlation between *PLXNA3* cytoplasmic domain variants (exons 22 to 32) and more severe neurodevelopmental disorder phenotypes ($P < 0.05$).

Conclusions: We report 14 boys with maternally inherited, hemizygous *PLXNA3* variants and a range of neurodevelopmental disorders suggesting a novel X-linked intellectual disability syndrome. Greater understanding of *PLXNA3* variant pathogenicity in humans will require additional clinical, computational, and experimental validation.

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Introduction

Neurodevelopmental disorders are complex and diverse disorders of the developing human brain. Clinical subclassification of neurodevelopmental disorders includes intellectual disabilities (ID) and autism spectrum disorders, which together (“autism/ID”) have a prevalence of approximately 3% to 5% of the population.^{1,2} Primary (*genetically determined*) neurodevelopmental disorders lead to secondary (*functionally defined*) neurodevelopmental disabilities, which cause a range of neurological impairments as well as immeasurable humanistic and economic burden.³

A genetic etiology can be identified in over half of all neurodevelopmental disorders.⁴ The integration of genomics into medical practice and the discovery of molecular diagnostic information has transformed the understanding of neurodevelopmental disorders. Over 2600 genes have been linked to autism/ID phenotypes according to multiple databases including Online Mendelian Inheritance in Man (OMIM), the Human Gene Mutation Database (HGMD), Human Phenotype Ontology (HPO), and the Simons Foundation Autism Research Initiative (SFARI).^{5–8}

With the ongoing expansion and curation of human genetic variation databases, genetic testing of individuals with autism/ID phenotypes is providing more accurate diagnostic information for affected families and more research potential for the science community. However, genetic testing frequently identifies genetic variants of uncertain clinical significance. Such is currently the case for genetic variants within the family of nine plexin (*PLXN*) genes, whose protein products act as transmembrane receptors for semaphorin signaling proteins in the intracellular Ras GTPase activation in the developing nervous system.^{9–12} Within the plexin family, *PLXNA3* (OMIM 300022; NCBI Reference Sequence: NM_017514.5) is located on the X chromosome and encodes plexin-A3. Investigation of plexin-A3 knockout mice shows that plexin-A3 contributes to *Sema3F* and *Sema3A* signaling and regulates the development of hippocampal axonal projections *in vivo*.¹³ This plexin-A3 protein is the most widely expressed in the fetal brain

and is also involved in cell motility, fasciculation, branching, and synapse formation.^{14,15}

Currently, there is not enough open-access patient data to provide quantitative information about *PLXNA3*. We identified a *PLXNA3* variant in a teenage boy with unexplained severe ID and sought other boys with *PLXNA3*-related neurodevelopmental disorder phenotypes through two large clinical genetic variation databases (GeneDx and GeneMatcher).¹⁶ The primary aim of this preliminary clinical study was to describe neurodevelopmental and neurocognitive phenotypes in boys with hemizygous *PLXNA3* variants. The secondary aim was to evaluate possible *PLXNA3* genotype-phenotype correlations.

Methods

Clinical data

We performed preliminary phenotyping on patients with rare hemizygous *PLXNA3* variants. Patients were identified through GeneDx and the global data exchange platform, GeneMatcher.^{16,17} We included patients with rare *PLXNA3* variants reported as variants of uncertain significance in accordance with the 2015 American College of Medical Genetics and Genomics and the Association for Molecular Pathology standards and guidelines.¹⁸

Case reports

We describe the proband in this study (Patient 1), who is a 17-year-old boy with longstanding severe autism/ID and a hemizygous *PLXNA3* variant identified on a trio GeneDx “Autism/ID Xpanded Panel,” which uses exome capture, next-generation sequencing, and targeted analysis of over 2000 genes associated with autism spectrum disorders and/or intellectual disability.^{19,20} A formal case study was developed through family, caregiver, and teacher interviews; medical record reviews; and home and school observation sessions as a part of a medical school research project

(J.L.S.). We also describe a fetal subject that was excluded from the clinical outcome analysis.

Case series

Data-sharing through GeneDx and GeneMatcher

All patients with *PLXNA3* variants in this study were either tested at GeneDx or identified through the global data exchange platform, GeneMatcher.^{16,17} All GeneDx patients were identified following the “Autism/ID Xpanded” panel or exome sequencing performed by GeneDx (Gaithersburg, MD, USA) using the Clinical Research Exome kit (Agilent Technologies, Santa Clara, CA) or the IDT xGen Exome Research Panel v1.0 (Integrated DNA Technologies, Coralville, IA). All GeneDx-identified *PLXNA3* variants were reported as variants of uncertain significance in accordance with the 2015 American College of Medical Genetics and Genomics and the Association for Molecular Pathology standards and guidelines.¹⁸

Data collection

Participating physicians and genetic counselors of *PLXNA3* patients were contacted through GeneMatcher. Clinical data were obtained from the collaborating clinicians through the completion of a questionnaire, which allowed acquisition of clinical information including the reason for referral, relevant medical and neurodevelopmental histories, pertinent physical findings, clinical diagnoses, estimated level of cognitive functioning (i.e., mild, moderate, or severe intellectual disabilities), genetic test results, neuroimaging results, and any other pertinent laboratory findings. Informed consent for genetic testing was obtained from parents by their care team (Duo or Trio analysis) before testing. The research protocol (Exempt Research [no identifiers recorded during the creation of a de-identified data set] (45 CFR 164.502(d)(1))) was approved by two institutional review boards (IRBs) (University of Connecticut and Connecticut Children's).

Clinical outcome analysis: exclusion criteria

All boys in this study, except for the fetus in case report 2, had maternally inherited *PLXNA3* variants. For the clinical study analysis, we excluded the fetal subject with a *de novo* *PLXNA3* variant because of the absence of phenotype and neurodevelopmental outcome data.

Variant annotation, in silico, and gnomAD analysis

Variants were annotated based on *PLXNA3* transcript NM_017514. *In silico* analyses for the missense variants were completed using Polyphen, Provean/Sift, Mutation Taster, and Combined Annotation-Dependent Depletion (CADD) to determine the likelihood of pathogenicity for each *PLXNA3* variant. The *in silico* analysis for the intronic insertion/splice variant was analyzed using Alamut. Predicted domain architecture information for the corresponding amino acids in the protein was retrieved from databases SMART, Prosite, InterPro, Pfam, and Regul@tionSpotter. The *PLXNA3* gene (search by genomic region: chrX:153,688,496-153,701,989 [GRCh37/hg19]) was analyzed from gnomAD (v2.1.1) database (<https://gnomad.broadinstitute.org/>), which contains genetic variants from 125,748 exome sequences and 15,708 genome sequences.²¹ Annotation of variants was performed with the Ensembl Variant Effect Predictor for human genome assembly GRCh37.

Consent to participate

According to the stipulations of the IRBs at Connecticut Children's (IRB#19-075) and University of Connecticut (IRB#: 19X-193-

1), informed consent to participate in the study was obtained from the parent of the proband (Patient 1) and the parents of the fetus. No personal identifiable information was gathered from the other subjects, and the patients themselves were not in contact with the primary researcher.

Consent for publication

Consent for publication was obtained from the parent of the proband in accordance with the Connecticut Children's and University of Connecticut IRBs.

Results

Clinical manifestations

Proband description

The proband for this study (Patient 1) is a 17-year-old adolescent boy diagnosed with severe ID and an overlapping diagnosis of autism. He was born at term with macrosomia but without other prenatal or perinatal concerns. Cleft palate, torticollis, and low muscle tone were noted at birth. Attainment of developmental milestones lagged in all domains. Delayed gross motor development was remarkable for rolling at age six months, independent walking around three years, and running, jumping, and tricycle riding by grade school age. Maximally attained functional fine motor skills include self-feeding using weight-modified utensils, scribbling with palmar pencil grasp, and semi-independent teeth brushing, buttoning, and zipping. Limited communication skills in childhood were enhanced through picture exchange communication systems. Minimal sign language and spoken words emerged in later childhood with an estimated vocabulary of approximately 50 meaningful intelligible words at age 17 years. Estimated intelligence quotient is <30. He cannot count or read. Out-of-context short-phrase mimicry and repetitiveness (such as “I'm mad”) are common. Vision and hearing are normal. He has limited attention except for fixation on certain objects (such as balls). He responds to positive reinforcement behavioral modification and demonstrates empathy. Bouts of unprovoked screaming and aggressive, intimidating, and self-injurious behaviors during childhood necessitated a paraprofessional aide through state developmental disability services. By midadolescence, he has normal gross motor skills and is a proficient swimmer. He shows diminished pain responses and nonspecific sleep disturbances. Generalized seizures beginning in late infancy were well-controlled with valproic acid and lamotrigine, and his electroencephalography normalized. Risperidone was prescribed due to aggressive behavior. Genetic testing showed hemizygoty for a c.5023A>G (p.Thr1675Ala) *PLXNA3* variant—reported as a variant of uncertain significance. Variant segregation analysis showed heterozygoty for the c.5023A>G variant in the mother and an absence of this variant in the father and healthy brother (Tables 1–4).

Fetus description

This male fetus spontaneously aborted at 18 weeks' gestation secondary to hydrops fetalis with intrauterine fetal death. The mother was a 34-year-old G5P10SA4L1 woman of Polish/Italian descent, and the father was 39 years old and of Italian/Polish/Ukrainian descent. The otherwise healthy nonconsanguineous parents have normal karyotypes and no extended family history of multiple miscarriages, congenital abnormalities, or inherited conditions. The mother's maternal half-brother was diagnosed with ADHD, dyslexia, and learning disabilities. This fetus was the fourth male loss after the mother's fifth pregnancy; the parents have a healthy daughter. In the three previous male fetus losses, no

obvious cause for the recurrent losses could be identified. Chromosomal microarrays were normal for the third and fourth losses. The first male fetus loss was in the first trimester, and subsequent losses were in the second trimester. Pathology investigation of the second loss suggested ascending infection. This fetal autopsy showed extensive maceration, possible clinodactyly; fragments of kidney, adrenal, heart, liver, and lung with extensive autolysis; fragmented placenta, with a markedly hypercoiled umbilical cord (10 turns per 10 cm [normal, 1 to 3]); and features of intrauterine retention post-fetal death, including villous vascular stromal karyorrhexis. Exome sequencing showed hemizygoty for a *de novo* c.4970G>A (p.Arg1657Gln) *PLXNA3* variant.

Data-sharing

From April 2019 to June 2020, GeneMatcher identified 70 candidate patients with *PLXNA3* variants. After describing our proband (patient 1), we collaborated with genetic counselors and physicians caring for the 13 additional boys and single male fetus selected among the 70 identified patients with *PLXNA3* missense variants. Patients 1, 4, 5, 6, 8, 9, 10, 11, 12, and 13 and the fetus were tested at GeneDx, and Patients 2, 3, 7, and 14 were identified through GeneMatcher (tables). All GeneDx-identified variants were classified as variants of uncertain significance.

Phenotype and clinical findings

In this cohort of 14 boys with X-linked inherited *PLXNA3* variants, we found a range of neurodevelopmental disorders and other clinically defined diagnoses (Tables 1 and 2). All *PLXNA3* patients had a clinical diagnosis of autism or ID, or both, which was the original indication for performing a GeneDx autism/ID panel. Seven subjects (50%) had moderate-severe intellectual disabilities, and the other seven were described as having mild-moderate intellectual disabilities. All subjects except one (Patient 5) had an autism diagnosis. All subjects showed speech and language impairments. The most common additional clinical diagnoses were attention-deficit/hyperactivity disorder or other behavioral problems (10 of 14 [71%]) and fine motor impairments (13 of 14 [92%]) (Table 2). Six patients (43%) had a history of seizures. All head circumference measurements were in the normal range. Neuroimaging was performed in 11 patients, six of whom had no remarkable findings. In

the five boys with remarkable neuroimaging studies, no common pattern of abnormal neuroanatomic findings was apparent (Table 1 and Discussion).

Molecular analysis

All *PLXNA3* patients in this cohort were identified by autism/ID panel testing or exome sequencing (Table 3). We identified nine novel *PLXNA3* variants. A genotype-phenotype correlation could be suggested by the observation that all six *PLXNA3* patients with variants coding for amino acid changes in the Plexin A3 cytoplasmic domain (exons 22–32) showed severe neurodevelopmental disorder phenotypes (two-tailed Fisher’s exact test, *P* = 0.0097). None of the variants reported in this study were found in ClinVar (NM_017514.5). Nine of the 14 variants from this study were not found in the gnomAD database. Four variants were present with an allele count of one and an allele frequency <1:100,000.

In silico analysis

Nine patients (64%) showed probably damaging/deleterious/disease-causing results in either all four *in silico* prediction models or the Alamut prediction model, and 13 subjects (93%) showed these results in at least one of the four *in silico* prediction models (Table 4). Of the nine patients with all four models or the Alamut model predicting pathogenicity, six had severe ID (67%). The single patient (patient 8) who had benign *in silico* prediction models also had a novel Plexin-A3 exon 13 extracellular domain variant (c.2494C>A/p.Gln832Lys) that correlated with a mild intellectual disability phenotype.

Discussion

Plexins are transmembrane receptors for semaphorin ligands that regulate multiple processes in the developing nervous system including cell migration and polarization, repulsive and attractive axonal guidance, laminar segregation, neuronal maturation, and apoptosis through intracellular Ras GTPase-activating protein (Ras-GAP) activation.^{9-12,15,22,23} Over 30 semaphorins are categorized into eight classes, and the nine total plexins are grouped into four subfamilies (A to D; plexin-A1-A4, B1-B3, C,

TABLE 1. Clinical Findings in 14 Boys With Neurodevelopmental Disorders and *PLXNA3* Variants

| Patient | Age (Years) | Seizures | Brain MRI | Family History |
|-------------|-------------|----------|-----------|--|
| 1 (Proband) | 17 | Yes | U | Healthy brother; <i>PLXNA3</i> negative |
| 2 | 11 | Yes | U | None |
| 3 | 6 | No | ** | Healthy older sister and younger brother (not tested) |
| 4 | 7 | Yes | ** | Sister with ID |
| 5 | 3 | No | U | Brother died as stillbirth; sister LD |
| 6 | 8 | No | U | Healthy brother (not <i>PLXNA3</i> tested); identity by descent |
| 7 | 14 | No | NA | Sister with autism |
| 8 | 25 | Yes | U | Brother with mild autism, <i>PLXNA3</i> negative |
| 9 | 11 | No | U | PKD, congenital deafness |
| 10 | 2 | No | ** | None |
| 11 | 16 | Yes | ** | Healthy brother; <i>PLXNA3</i> negative |
| 12 | 18 | Yes | ** | None |
| 13 | 13 | No | NA | None |
| 14 | 5 | No | NA | Brother with speech delay; twin sisters died with pulmonary insufficiency of prematurity |

Abbreviations:
 ID = Intellectual disability
 LD = Learning disability
 MRI = Magnetic resonance imaging
 NA = Not applicable
 PKD = Polycystic kidney disease
 U = Unremarkable/normal
 **: See Discussion for details.

TABLE 2.
Developmental Findings in 14 Boys With Neurodevelopmental Disorders and *PLXNA3* Variants

| Patient | Intellectual Disability Level | ASD or Other Behavioral Diagnoses | Motor Impairment (GM/FM) | Language Impairment | Prenatal/Perinatal Findings |
|-------------|-------------------------------|-----------------------------------|--------------------------|---------------------|--------------------------------|
| 1 (Proband) | Severe | ASD; ADHD, aggressive | -/+ | Moderate-severe | LGA; cleft palate; torticollis |
| 2 | Moderate | ASD; ADHD, aggressive | +/+ | Moderate-severe | Unknown |
| 3 | Mild-moderate | ASD; ADHD | +/+ | Mild-moderate | Torticollis |
| 4 | Mild | ASD; ADHD, aggressive | -/+ | Moderate-severe | Unknown |
| 5 | Mild-moderate | Cognitive rigidity | +/+ | Moderate | Chylothorax |
| 6 | Severe | ASD; Aggression | -/+ | Moderate-severe | Umbilical cord prolapse |
| 7 | Mild | ASD; OCD | -/- | Mild | IUGR |
| 8 | Mild | ASD; ADHD, anxiety | -/+ | Moderate-severe | None |
| 9 | Mild-moderate | ASD; ADHD, anxiety, aggressive | +/+ | Moderate-severe | None |
| 10 | Severe | ASD | +/+ | Moderate-severe | IUGR |
| 11 | Severe | ASD; ADHD | +/+ | Moderate-severe | None |
| 12 | Severe | ASD | +/+ | Severe | None |
| 13 | Severe | ASD | +/+ | Moderate-severe | None |
| 14 | Moderate-Severe | ASD | -/+ | Severe | None |

Abbreviations:

ADHD = Attention-deficit/hyperactivity disorder
 ASD = Autism spectrum disorder
 FM = Fine motor
 GM = Gross motor
 ID = Intellectual disabilities
 IUGR = Intrauterine growth retardation
 LGA = Large for gestational age
 OCD = Obsessive-compulsive disorder

and D). *PLXNA3* is located on chromosome Xq28, and its major transcript consists of 33 exons (Fig 1). The encoded plexin-A3 protein is the most widely expressed plexin in sensory, sympathetic, and hippocampal neurons during early neurodevelopment when neurons are most responsive to semaphorins. Plexin-A3 is a specific receptor for the semaphorins SEMA3A and SEMA3F, which bind with the coreceptor neuropilin-1 to modulate its cytoplasmic domain for the activation of downstream intracellular signaling events (Fig 2).²⁴ Plexin-A3 knockout mice show

direct functional evidence for signaling roles such as guidance of sympathetic and hippocampal axons *in vivo*.¹³ In zebrafish embryos, *Plxna3* mutations act as an essential component for *Sema3a1* signaling for fasciculation and target selection of the extending axons of motoneurons.²⁵ Although disruptions in axonal outgrowth and pathfinding are highly relevant to the broad autism/ID phenotype, the functional importance of *PLXNA3* missense variants and their clinical manifestations in human neurodevelopment are yet to be fully explained.

TABLE 3.
Molecular Genetic Findings in 14 Boys With Neurodevelopmental Disorders and One Male Fetus With *PLXNA3* Variants (Reference Transcript NM_017514)

| Patient | Coding DNA Sequence Variant | Protein Sequence Variant | Splice Sites Abrogation | Exon: Chromosome Position | VEP Annotation | Other Genetic Findings |
|-------------|-----------------------------|--------------------------|-------------------------|---------------------------|----------------|--|
| 1 (Proband) | c.5023A>G | p.T1675A | No | 30:153698821 | Missense | 2 small maternally inherited duplications, VUS |
| 2 | c.653T>C | p.L218S | No | 3:153689497 | Missense | - |
| 3 | c.1143G>C | p.Q381H | No | 4:153690476 | Missense | 13q12.12 deletion, VUS |
| 4 | c.1400T>A | p.L467H | No | 5:153691816 | Missense | Maternally inherited, pathogenic GLMN variant (c.743dupT, p.Leu248Phefs*14) |
| 5 | c.2041G>A | p.E681K | Yes | 10:153693209 | Missense | Microarray: regions of homozygosity 251 Mb |
| 6 | c.2342C>G | p.A781G | Yes | 13:153694000 | Missense | - |
| 7 | c.2363C>T | p.A788V | No | 13:153694021 | Missense | - |
| 8 | c.2494C>A | p.Q832K | No | 13:153694152 | Missense | - |
| 9 | c.3407T>C | p.L1136P | No | 19:153695780 | Missense | 76-kb duplication on chr 10q24.32 |
| 10 | c.4250C>T | p.T1417I | No | 24:153697035 | Missense | Maternally inherited homoplasmic MT-TC variant m.5786dupT- |
| 11 | c.4601G>A | p.R1534H | Yes | 27:153697728 | Missense | 520-kb gain-of-material VUS at 3p21.1 (includes <i>TKT</i> and exons 1-8 of <i>CACNA1D</i>) |
| 12 | c.4616C>G | p.T1539S | Yes | 27:153697743 | Missense | Heterozygous VUS in <i>CTNND2</i> (c.859C>T, p.P287S) |
| 13 | c.5041C>A | p.H1681N | No | 30:153698839 | Missense | 48-kb VUS duplication on chr 7p14.1 with 12.13% regions of homozygosity |
| 14 Fetus | c.5156+1G>T | - | Yes | | Intron | Spontaneous miscarriage at 18 weeks' gestation, see text |
| | c.4970G>A | p.R1657Q | No | | Missense | - |

Abbreviations:

MT-TC = Mitochondrial
 N = Normal/neutral
 NMV = No matching variants
 VEP = Variant Effect Predictor
 VUS = Variant of uncertain significance

TABLE 4.
In Silico and gnomAD Analysis in 14 Boys With Neurodevelopmental Disorders and One Male Fetus With PLXNA3 Variants

| Patient | In Silico Analysis/Functional Effect Prediction | | | | CADD Score (PHRED-like) | ClinVar NM_017514.5 | Allele Frequency in gnomAD* [†] Hemizygotes |
|-------------|---|-----------------|-----------------------|--------------------|-------------------------|---------------------|--|
| | PolyPhen-2 HumVar | Mutation Taster | Provean (Cutoff -2.5) | Sift (Cutoff 0.05) | | | |
| 1 (Proband) | 0.969 PD | 0.999 DC | -4.56 D | 0.002 D | 25.3 | NMV | 5.49e-6/0 |
| 2 | 0.627 PD | 0.917 DC | -0.75 N | 0.32 T | 22.8 | NMV | NMV |
| 3 | 0.907 PD | 0.999 DC | -3.28 D | 0.022 D | 25.3 | NMV | 2.68e-5/1 |
| 4 | 0.125 B | 0.818 DC | -2.9 D | 0.25 T | 21.7 | NMV | NMV |
| 5 | 0.661 PD | 0.999 DC | -2.69 D | 0.041 D | 30 | NMV | NMV |
| 6 | 0.155 B | 0.999 DC | -3.11 D | 0.001 D | 25 | NMV | NMV |
| 7 | 0.833 PD | 0.999 DC | -2.74 D | 0.263 T | 24.3 | NMV | 5.54e-6/1 |
| 8 | 0.004 B | 0.51 PP | -1.01 N | 0.454 T | 16.59 | NMV | 5.70e-6/0 |
| 9 | 0.997 PD | 0.999 DC | -5.0 D | 0.003 D | 27.8 | NMV | NMV |
| 10 | 0.959 PD | 0.999 DC | -4.71 D | 0.001 D | 27.2 | NMV | NMV |
| 11 | 1.00 PD | 0.999 DC | -4.29 D | 0.001 D | 32 | NMV | NMV |
| 12 | 0.003 B | 0.935 DC | 0.32 N | 0.035 D | 22.4 | NMV | 5.49e-6/0 |
| 13 | 0.969 PD | 0.999 DC | -5.99 D | 0.026 D | 24.8 | NMV | NMV |
| 14 | Alamut score -1[‡] | - | - | - | - | NMV | NMV |
| Fetus | 1.0 PD | 0.999 DC | -3.57 D | 0 D | - | NMV | 5.58e-6/0 |

Abbreviations:

B = Benign

CADD = Combined Annotation-Dependent Depletion

D = Deleterious/damaging

DC = Disease causing

N = Normal/neutral

NMV = No matching variants

PD = Probably damaging

PP = Prediction polymorphism; Slice sites, alteration within used splice site, likely to disturb normal splicing

T = Tolerated

* Global allele frequency in gnomAD (v2.1.1), N = 141,456 exomes and genomes.

[†] Number.

[‡] Predicted to cause abnormal gene splicing by destroying the canonical splice donor site in intron 30.

We describe 14 boys with maternally inherited *PLXNA3* variants and a range of neurodevelopmental disorder phenotypes, which suggests the possibility of variable expressivity in *PLXNA3* disorders. All subjects showed impairments in cognition and verbal communication with varying degrees of severity. The composite impression of the boys in this cohort did not suggest a physically recognizable phenotype, even if Patient 1 had a cleft palate, and in addition mild nonspecific facial features were described in four patients including hypotonic face with a bowed upper lip (Patient 2); synophrys, upturned ear lobules, and a prominent nasal bridge (Patient 6); a narrow face (Patient 8); and hooded eyelids with focal patchy hyperpigmentation (Patient 14).

Similarly, we did not observe a recurring neuroimaging prototype in this study. Of the 11 *PLXNA3* patients who had neuroimaging, six were reportedly unremarkable and five showed

anomalies including a thin corpus callosum with possible dysplastic splenium and posterior white matter (patient 3), periautrial white matter and centrum semiovale with white matter volume loss (Patient 4), large lateral ventricles and cerebrospinal fluid spaces (patient 10), dysmorphic medial temporal lobes and hippocampal architecture with adjacent subcortical white matter volume loss (patient 11), and low white matter volume (patient 12). In general, there is limited neuroimaging understanding of how such axonal guidance disorders in human case reports may result in normal or abnormal tissue morphology. In plexinA1-deficient (knockout) mice (similar to plexinA3, plexinD1, and L1CAM), immunohistochemistry in embryonic brains demonstrates the essential role of plexin-mediated cell adhesion of cingulate axons and the midline crossing of callosal axons during corpus callosum development.²⁶

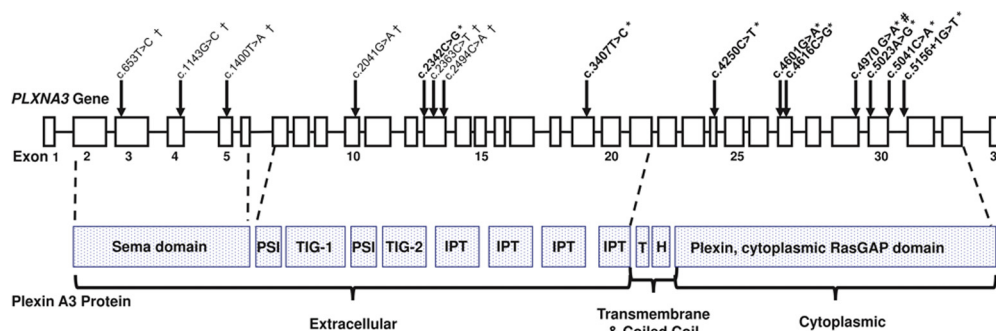


FIGURE 1. Representation of *PLXNA3* and its plexinA3 protein structure. The 33 exons of the *PLXNA3* gene are represented by boxes; introns are represented by a solid line. Distribution of novel *PLXNA3* variants and their exon distribution (arrows) corresponding to extracellular, transmembrane, and cytoplasmic plexinA3 domains. Patients with a *PLXNA3* variants and severe autism/intellectual disabilities phenotype are highlighted in bold followed by an asterisk (*). Patients with mild-moderate autism/intellectual disabilities phenotype are not bolded and followed by a cross (†). The fetus with the c.4970G>A variant was excluded from analysis (#) (see text). IPT, immunoglobulin-like fold shared by plexins and transcription factors; PSI, Plexin, Semaphorin, and Integrin domains; T, transmembrane; H, helix coiled coil; TIG, Immunoglobulin (Ig) domain shared by Plexins and Transcription factors. The color version of this figure is available in the online edition.

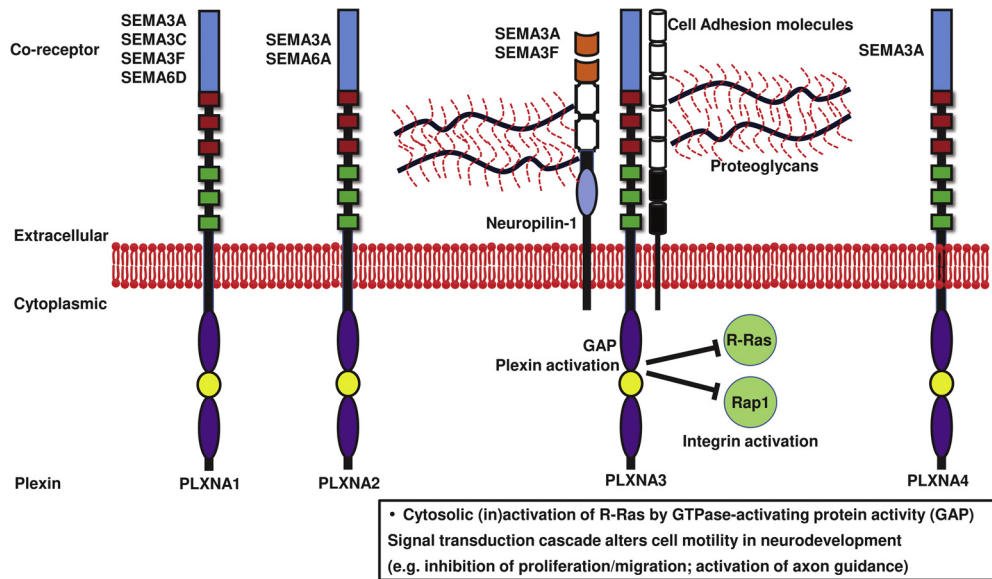


FIGURE 2. Schematic representation of the extracellular, transmembrane, and cytoplasmic regions of the four PLXNA semaphorin receptor structures and their corresponding semaphorin subtypes. R-Ras, Ras-related protein; Rap-1, Ras-proximate-1. The color version of this figure is available in the online edition.

In this preliminary study, all 14 boys included for analysis had documented maternally inherited *PLXNA3* variants. The likely pathogenicity of *PLXNA3* is suggested by the absence of such variants in the unaffected, neurologically healthy brothers of Patients 1 and 11 in this study as well as the likely projected deleterious/damaging and disease-causing consequences by *in silico* predictors. The absence of clinical reports about these *PLXNA3* variants and the absence or rarity of these variants in the Genome Aggregation Database (gnomAD) further supports pathogenicity.

The male fetus described in this report had a *de novo* *PLXNA3* variant. Future studies should examine the significance of *de novo* *PLXNA3* variants and the role of germline mosaicism in plexin-related developmental disorders. No female subjects were reported in this cohort, but as genomic databases grow the possibility of finding girls with neurodevelopmental disorders and heterozygous *PLXNA3* variants with random X inactivation is likely.

An endocrinology study of patients with hypogonadotropic hypogonadism found loss-of-function variants in *SEMA3F* and *PLXNA3*, where six of the seven reported patients with *PLXNA3* variants had affected extracellular ligand and coreceptor interactions based on the structural model of the protein (i.e., three in the SEMA binding domain, two in the Plexin-Semaphorin-Integrin [PSI2] domain, and one in the Ig domain [IPT3 region])²⁷ (see protein domains illustrated in Fig 1). The sole hypogonadotropic hypogonadism subject who had a cytoplasmic *PLXNA3* variant was a female patient.²⁷ None of the boys in our study were diagnosed with hypogonadotropic hypogonadism.

In our cohort, the most severe neurodevelopmental disorder phenotypes were associated with *PLXNA3* cytoplasmic domain variants (exons 22 to 32 in Fig 1). Although the exact reason for this apparent genotype-phenotype correlation remains uncertain, plexins are known to have highly conserved cytoplasmic domains that contain GAP and GAP-like motifs for regulating molecules such as R-Ras, a RAS family member whose function includes regulation of integrin-dependent adhesion and other downstream intracellular processes^{28,29} (Fig 2). In mouse embryos, deletion of the highly conserved plexin cytoplasmic domain creates a dominant-negative effect on their response to the repulsive Sema3A signal.³⁰ Such findings demonstrate the repulsive (or inhibitory) effects of Sema3A that are mediated by plexin/neuropilin

complexes and require the large and highly conserved cytoplasmic plexin domain to activate the downstream signal transduction machinery leading to the cell-type specific repulsive and attractive effects on axons.

Other transmembrane proteins can act as coreceptors or receptors for semaphorins and neuropilin/plexin receptor complexes, such as cell adhesion molecules, integrins, and proteoglycans such as heparan sulfate (perlecan), chondroitin sulfate, and keratan sulfate (lumican) proteoglycans^{12,31–33} (Fig 2). The relationship between keratan sulfate proteoglycan, the most abundant glycosaminoglycan chain in the developing nervous system, and semaphorin-plexin signaling remains to be fully defined.³⁴ Keratan sulfate is an essential extracellular matrix fibrillogranular protein that guides axonal trajectory in the developing central nervous system by surrounding axonal fascicles to prevent axonal exit before they reach their programmed destination and by preserving the purity of axonal contents of white matter tracts.³⁵ Keratan sulfate ensheathment of axons within central tracts occurs several months before the initiation of myelination of those axons. An example is alobar/semilobar holoprosencephaly, where keratan sulfate ensheathes individual axons in the developing fetal brain, further isolating them and perhaps helping to explain why up to 40% of infants with holoprosencephaly do not have epilepsy despite the finding of severe dyslamination of their cerebral cortices.³⁶

In addition to their essential roles in prenatal neurogenesis, semaphorin/plexin complexes have been implicated in various postnatal-onset disorders and diseases including gliogenesis, neurodegeneration, and malignancy.^{15,37} *PLXNA4* variants have been linked to Alzheimer and Parkinson diseases.^{38,39} The effects of plexin genes are demonstrated in the normal distribution of human intelligence ranging from presumed pathogenic variants in certain intellectual disabilities to enriched expression of plexin genes in extremely high intelligence.⁴⁰

The limitations of this study include the challenges of any observational, cross-sectional, and retrospective study design. We acknowledge the challenge of finding a collective *PLXNA3* patient phenotype through the clinical descriptions by multiple clinicians with diverse training in genetics and pediatric neurology. Seven *PLXNA3* patients in this study had additional genetic or genomic variants. One patient (Patient 5) had a maternally inherited,

pathogenic *GLMN* variant, and six patients had genomic variants of uncertain significance including a small chromosome 13q12.12 deletion (Patient 4); a 76-kb duplication on chromosome 10q24.32 (Patient 10); a mitochondrial (MT-TC) variant (patient 11); a 520-kb duplication on chromosome 3p21.1 (Patient 12), which includes *TKT* and exons 1 to 8 of *CACNA1D*; a heterozygous *CTNND2* variant (patient 13); and a 48-kb chromosome 7p14.1 duplication (Patient 14) with 12% regions of homozygosity (suggesting consanguinity). The effect of these seven co-occurring variants on the range of intellectual disabilities and other features in this cohort is uncertain (Tables 1 and 2).

Although the proportion of X-linked genes involved in neurodevelopmental disorders is recognized to be higher than autosomal genes, less is known about the specific effects of many rare X-linked variants in complex traits such as cognition.⁴¹ The *PLXNA3* predicted deleterious missense variants in our subjects are either novel or have a very low allele frequencies (less than 1/10,000), and thus the power of the loss-of-function observed/expected upper bound fraction metric (currently estimated as 0.384 for *PLXNA3*) is limited. However, the probability of loss-of-function intolerance ($pLI \geq 0.9$) metric for *PLXNA3* predicts a high level of constraint.⁴² In addition, it is not known whether pregnancies in asymptomatic women carrying pathogenic loss-of-function variants lead to natural selection with higher rates of fetal loss (suggested by case report 2 in this study) or the reduction of such *PLXNA3* variants in genetic studies of postnatally diagnosed neurodevelopmental disorders such as autism/ID.

Thus, it remains uncertain whether all of the maternally inherited hemizygous *PLXNA3* missense variants in our cohort are unambiguously benign or deleterious. One subject in this study (Patient 8) may have a likely benign *PLXNA3* variant. Similarly, our review of the *PLXNA3* literature and the very small number of previously reported patients with high-frequency *PLXNA3* missense variants suggests some may be likely benign.⁴³

In summary, we describe a small cohort of boys with maternally inherited hemizygous *PLXNA3* variants and a range of neurodevelopmental disorders characterized by intellectual disabilities with codiagnosed autism, behavioral abnormalities, and epilepsy. These observations suggest a novel X-linked plexin intellectual disability syndrome. Additional clinical, computational, and neurobiological studies are needed to further elucidate *PLXNA3*-related developmental disorders especially in the setting of a familial X-linked inheritance pattern.

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