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Novel pathogenic genomic variants leading to autosomal dominant and recessive Robinow syndrome

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Abstract

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Author Contributions:

C.Z., J.R.L. and C.M.B.C.: designed the study and take responsibility for the integrity of the data and accuracy of the data analysis. C.Z., J.F.M., C.M.G., J.W., V.R.S. and C.M.B.C.: collected the data. J.E. and A.L.: performed genome sequencing and structural variant analysis. Z.C.A., S.N.J., D.M.M., and R.A.G.: performed exome sequencing and variant annotation. C.Z.: analyzed data and wrote the initial manuscript draft. J.R.L., V.R.S. and C.M.B.C.: provided critical input into the writing of the manuscript. All the authors: were involved in reviewing and revising the final submitted manuscript.

Disclosures

Baylor College of Medicine (BCM) and Miraca Holdings have formed a joint venture with shared ownership and governance of the Baylor Genetics (BG), which performs clinical microarray analysis and clinical exome sequencing. J.R.L. serves on the Scientific Advisory Board of the BG. J.R.L. has stock ownership in 23andMe, is a paid consultant for Regeneron Pharmaceuticals, and is a coinventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from molecular genetic and clinical genomics testing offered at BG. The other authors declare no competing financial interests.

Accession Numbers

The submission numbers for identified variants were deposited into ClinVar with the preliminary identifiers: SUB8111875. The dbGAP accession number for all exome sequences reported in this paper and for which informed consent for data sharing in controlled-access databases has been provided is dbGAP:phs000711.v5.p1

Robinow syndrome (RS) is a genetically heterogeneous disorder characterized by skeletal dysplasia and a distinctive facial appearance. Previous studies have revealed locus heterogeneity with rare variants in *DVL1*, *DVL3*, *FZD2*, *NXN*, *ROR2*, and *WNT5A* underlying the etiology of RS. The aforementioned 'Robinow associated genes' and their gene products all play a role in the WNT/planar cell polarity (PCP) signaling pathway. We performed gene-targeted Sanger sequencing, exome sequencing (ES), genome sequencing (GS) and array comparative genomic hybridization (aCGH) on four subjects with a clinical diagnosis of RS who had not had prior DNA testing. Individuals in our cohort were found to carry pathogenic or likely pathogenic variants in three RS related genes: *DVL1, ROR2* and *NXN*. One subject was found to have a nonsense variant (c.817C>T [p.Gln273*]) in *NXN in trans* with an ~1 Mb telomeric deletion on chromosome 17p containing *NXN*, which supports our contention that biallelic *NXN* variant alleles are responsible for a novel autosomal recessive RS locus. These findings provide increased understanding of the role of WNT signaling in skeletal development and maintenance. These data further support the hypothesis that dysregulation of the noncanonical WNT pathway in humans gives rise to RS.

Keywords

skeletal dysplasia; clinical diagnosis; deletion; missense; structural variant

Introduction

Robinow syndrome (Robinow et al., 1969), RS, is a genetically heterogeneous disorder that is characterized by skeletal dysplasia, genital hypoplasia, and a distinctive facial appearance, that includes frontal bossing, hypertelorism and a short nose. RS can segregate as an autosomal-recessive (AR) (RRS [MIM#268310]) or autosomal-dominant (AD) trait (DRS [MIM#180700]) (Mazzeu and Brunner, 2020). Previous studies have identified six genes in which pathogenic variants can cause RS. Two genes are associated with recessive forms or RRS, *ROR2* and *NXN,* whereas DRS results from heterozygous pathogenic variants in *WNT5A, FZD2, DVL1* and *DVL3.* Additionally, biallelic loss-of-function variants in *WNT5A* were reported in one patient with RS (Birgmeier et al., 2018).

DVL1 and *DVL3* are notable for small insertions and deletions (indels) that exclusively result in a −1 frameshift of the last coding exon. Collective evidence indicates that dysregulation of the noncanonical WNT planar cell polarity (PCP) pathway in humans gives rise to Robinow syndrome as all the RS associated genes and their protein products play a role in this specific pathway. RRS is caused by biallelic loss-of-function variant alleles (LoF) in *ROR2*, a transmembrane receptor essential for PCP signaling (Isao Oishi, 2003; Minami et al., 2010) or *NXN*, a gene that can act as a negative regulator of the WNT pathway (Funato et al., 2006; Funato et al., 2008). Biallelic LoF variants affecting *NXN* were recently found to cause RRS [MIM#618529]; however, only three patients, two of them siblings, have been reported to date (White et al., 2018).

Recently, *de novo* indels were found in two out of the three human paralogues of the Drosophila *dishevelled (dvl)* gene, *DVL1* and *DVL3*. DVL1 and DVL3 are key downstream mediators of the WNT pathway via interaction with ROR2 (Gao and Chen, 2010;

Witte et al., 2010). These pathogenic variants are protein-truncating and clustered in the ultimate and penultimate exons, which is a frequent cause of DRS (DRS2 [MIM#616331], DRS3[MIM#616894])(Bunn et al., 2015; White et al., 2015; White et al., 2018; White et al., 2016). Invariably, pathogenic variants affecting *DVL1* and *DVL3* are predicted by conceptual translation via the genetic code to consistently generate a −1 frameshifting encoded protein with a premature termination codon (PTC) in the last exon. This type of specific variant allele, estimated to affect 33% of individuals with a diagnosis of RS (White et al., 2018), produce a mutant mRNA that is predicted to escape degradation via the nonsense mediated decay (NMD) RNA surveillance system (Coban-Akdemir et al., 2018; Danyel et al., 2018; White et al., 2015; White et al., 2016). As a result, mutant DVL1 and DVL3 proteins replace their normal C-terminus with a highly basic mutant C-terminal tail. Besides *DVL1* and *DVL3*, heterozygous missense mutations in *WNT5A,* the extracellular soluble ligand of ROR2, account for ~9.5% of individuals with a diagnosis of RS (DRS1 [MIM#180700])(Person et al., 2010; Roifman et al., 2015; White et al., 2018). In addition, variants in *FZD2,* including a recurrent missense and multiple truncating variants, have been found to cause ~14% of RS (White et al., 2018). FZD2 is a highly conserved seven-pass transmembrane protein of the Frizzled family of membrane receptors which function as WNT receptors and co-receptors.

In 2018, a Robinow Syndrome Family Conference was held in Houston, TX. Thirteen subjects (Supplemental Table 1) diagnosed as Robinow syndrome or Robinow-like phenotypes were recruited and evaluated clinically by specialists in a multidisciplinary team. Four out of 13 patients involved in this conference did not have a molecular diagnosis from previous investigations. Considering that the aforementioned Robinow genes all play a role in the WNT/PCP signaling pathway, we sought to search for variants in known RS genes as well as discover additional genes that may dysregulate the noncanonical WNT pathway in these four clinically diagnosed patients.

Methods

Subjects

Subjects were recruited from the 2018 Robinow Syndrome Family Conference in Houston, TX (Supplemental Table 1). All subjects (n=13) were clinically evaluated on the same day by a multidisciplinary team consisting of specialists in medical genetics, urology, psychiatry, and plastic surgery. This study was approved by the institutional review board at Baylor College of Medicine (IRB protocol no. H-43246). From the total of 13 enrolled subjects, four did not have a molecular diagnosis concluded from previous studies. Supplementary Table 1 shows the molecular findings for all 13 subjects.

Sanger sequencing

The molecular workflow of research samples from patients clinically diagnosed as RS is shown in Supplemental Figure 1. We performed a combination of gene-targeted Sanger sequencing, exome sequencing (ES), genome sequencing (GS) and array comparative genomic hybridization (aCGH) on 4 subjects (BAB10151, BAB9136, BAB10973 and BAB14232) with a clinical diagnosis of RS who had not had prior DNA testing. For

BAB9136 and BAB14232, we performed Sanger sequencing of all *ROR2* exons; primers are shown in Supplemental table 2.

Exome sequencing

ES was performed on BAB10151 and BAB10973 at the Baylor College of Medicine-Human Genome Sequencing Center (BCM-HGSC) as previously reported (White et al., 2018). Specific population frequencies of variants were obtained from gnomAD v.2.1.1 (https://gnomad.broadinstitute.org/) (Karczewski et al., 2020). Variants were reclassified by American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015; Riggs et al., 2020). Criteria of ACMG classification are shown in Supplemental Table 3.

Genome sequencing

GS was performed on BAB10973 as described (Lindstrand et al., 2019). Structural variants were analyzed using the FindSV pipeline, which combines CNVnator V0.3.2 (Abyzov et al., 2011) and TIDDIT V2.0.0 (Abyzov et al., 2011). The resulting variant call file (VCF) was annotated using variant effect predictor (VEP) 87 (McLaren et al., 2016).

RNA analysis

Total RNA was extracted from lymphoblastoid cell lines from BAB10151 using the polytron method. The qScript cDNA SuperMix (Quanta Biosciences) was used for cDNA synthesis. *DVL1* mRNA fragment was amplified using the primers and protocol as described (White et al., 2015).

Array comparative genomic hybridization

Customized aCGH in 2×400K format (AMADID# 085772, Agilent Technologies), which tiles the entire chromosome 17 (chr17:1–81195210, 1030 median probe space), was performed on genomic DNA isolated from blood obtained from subject BAB10973. Experimental steps of aCGH, including DNA fragmentation, DNA labeling and clean-up, array hybridization, array washing, and scanning were performed as previously described (Beck et al., 2019).

Results

Our experimental studies and analyses revealed novel pathogenic variants in three RS related genes: *DVL1, ROR2* and *NXN*. BAB10151 harbors a novel single nucleotide deletion, c.1556del, p.Gly519Aspfs*130, (GenBank: NM_004421.2) in *DVL1* (Table 1). This variant was not present in the maternal sample (father's sample was not available for testing). WT and mutant alleles are expressed in BAB10151 as observed in Sanger sequenced cDNA extracted from Epstein-Barr lymphoblastoid B cell lines (Supplemental Figure 2). Expression of the mutant mRNA is consistent with previous experimental observations from White et al. 2015 (White et al., 2015) indicating that this allele escapes efficient NMD.

BAB9136 was found to have compound heterozygous *ROR2* variants (Supplemental Figure 3) including a missense variant c.899G>T (p.Cys300Phe) in exon 6 and a single nucleotide

deletion c.990delC (p.Thr331Profs*114) affecting exon 7 (GenBank: NM_004560.3). The missense variant is not observed in gnomAD or ExAC databases, and the Combined Annotation Dependent Depletion (CADD) score is 28.1. The unaffected mother carries only one of the variants, the missense allele (c.899G>T [p.Cys300Phe]); the father was unavailable for segregation studies. TA cloning of exons 6 and 7 amplified as a single PCR product confirmed that the variants are present in trans in the patient sample (Supplemental Figure 3). BAB14232 was found to carry compound heterozygous missense variant alleles affecting *ROR2* (Supplemental Figure 4). One allele, c.904C>T (p.Arg302Cys, exon 6, CADD score: 27.6), was inherited from the father, BAB14234; in accordance with Mendelian expectations, the other variant allele c.1970G>A (p.Arg657His, exon 9, CADD score: 33) was inherited from the mother, BAB14233.

In the fourth patient, BAB10973, ES identified an apparent homozygous nonsense variant in *NXN* c.817C>T (p.Gln273*) (GenBank: NM_022463.4). The patient was adopted, and we had no information regarding consanguinity. *NXN* is located in chr17p13.3, heterozygous deletion of this region causes chromosome 17p13.3 deletion syndrome or Miller–Dieker lissencephaly syndrome (MDLS[MIM#247200]). The main phenotype includes core nervous system anomalies, seizure, and facial dysmorphism(Bi et al., 2009; Chen et al., 2013; Gu et al., 2015). To examine copy number at the *NXN* locus, we proceeded with investigations by digital droplet PCR and aCGH that revealed an approximately 1 Mb telomeric deletion of chromosome 17p (Figure 1) that includes *NXN* and 12 other genes (seq[GRCh37/hg19] del(17)(p13.3) chr17:g.pter_1026797del). This deletion does not include *PAFAH1B1* or *YWHAE* related to Miller-Diecker deletion syndrome, and no other genes in this region have been associated with disease in the heterozygous state. To confirm the estimated size based on aCGH and further reveal the breakpoint junction of this deletion, both GS and standard Sanger sequencing were performed (Figure 1E, 1F). As a result, read depth analysis of GS data also confirmed the 17p telomeric deletion (data not shown). The breakpoint junction included telomeric repeats suggesting either a subtelomeric deletion or a terminal deletion and single ended double stranded DNA (seDNA) whose end was healed by a telomerase mediated event. Parental samples were not available for further testing so we could not investigate inheritance of either mutational event.

Discussion

The genetic and clinical findings from four RS patients are consistent with the proposed biological mechanism for this disease. For *DVL1*, one individual was found to have a novel 1 bp deletion located in exon 14. *Dvl1*-null mice showed abnormal social behavior rather than skeletal defects (Lijam et al., 1997; Long et al., 2004), which in addition to our identified *DVL1* variants, strengthens the hypothesis that the pathogenic *DVL1* variants act as gain-of-function (GoF) rather than by loss-of-function (LoF) and haploinsufficiency (White et al., 2018). Consistent with previously reported patients (Bunn et al., 2015; White et al., 2015; White et al., 2018; White et al., 2016), this frameshift allele creates a large mutant C-terminal tail of 129 amino acids.

ROR2 variants have been identified by many previous studies of RS (van Bokhoven et al., 2000). In this study, a single nucleotide deletion c.990delC (p.Thr331Profs*114) and

a heterozygous missense variant at the position c.899G>T (p.Cys300Phe) were found in patient BAB9136 (Table 1). This novel rare variant c.899G>T is located in exon 6 where pathogenic variants have been frequently observed. Variants in exon 6 have been shown to affect a cysteine rich domain of ROR2 that is important for intracellular localization (Ali et al., 2007). Alterations in the amino acid structure in this domain may perturb correct folding of ROR2, potentially by a Cys requirement and for a Cys-Cys disulphide linkage and secondary structure formation which may affect the export of ROR2 from the endoplasmic reticulum to the plasma membrane. The second novel 1 bp deletion identified in this patient at c.990delC (p.Thr331Profs*114) was found in *trans*. This rare variant located in exon 7 of *ROR2* likely leads to an RNA that is degraded by the NMD RNA surveillance system, as predicted by our NMD prediction tool NMDescPredictor (https://nmdprediction.shinyapps.io/nmdescpredictor/) (Coban-Akdemir et al., 2018) resulting in a null allele.

An additional heterozygous missense variant located in exon 6 of *ROR2* was found in patient BAB14232 (c.904C>T [p.Arg302Cys]) (Table 1) and was recently reported in a fetus with RRS (Yang et al., 2019). In addition, a likely pathogenic missense variant c.1970G>A (p.Arg657His) in exon 9, with CADD score of 33, was observed in *trans* in BAB14232. Pathogenic variants in *ROR2* have been related to two distinct human disorders, RRS and autosomal dominant Brachydactyly type B (MIM# 113000). Ben-Shachar et al reported that distinct phenotypes and different inheritance patterns can potentially be explained by triggering or escaping NMD (Ben-Shachar et al., 2009). Different phenotypes may be determined by the relative degree of protein retention/degradation and the amount of mutant protein reaching the plasma membrane (Schwarzer et al., 2009). In summary, these findings support the initial hypothesis that RRS results from biallelic variants in *ROR2* causing LoF.

We also identified biallelic variants in *NXN* in one RS subject (Figure 1). Thus far, only three RS patients from two families, including affected siblings from the same family, have been reported to carry biallelic variants in *NXN* (White et al., 2018).The observation in an unrelated third family of a fourth patient with biallelic variants further supports *NXN* as an RRS gene. *NXN* encodes an oxidative stress response protein nucleoredoxin that is highly expressed in the developing limb bud of mice (Kurooka et al., 1997). NXN was found to be a negative regulator of the WNT pathway (Funato et al., 2006; Funato et al., 2008). Additionally, pull-down assays of mouse fibroblasts indicated that Nxn is a substantial interacting partner of Dvl1, particularly under oxidative conditions, often stimulated by growth factors (Funato et al., 2006; Sundaresan et al., 1995). The interaction between NXN and DVL is hypothesized to be a key regulatory mechanism to maintain a spatial or temporal balance between canonical and noncanonical WNT pathways during development (Funato et al., 2010).

It has been reported that the β-catenin-independent pathway can be activated by binding of Wnt5a to Frizzled2 (Fz2) with the help of Ror2 (Sato et al., 2010). Thus, it is possible that perturbation of this specific pathway, which is activated by WNT5A, FZD2 and ROR2 causes RS. Based on our experimental findings and previous published reports on Robinow syndrome, we adapted, modified and updated a representative model from White

et al, which illustrates the physical interactions between all identified Robinow-associated proteins with a role in the WNT/PCP pathway in humans (White et al., 2018) (Figure 2).

In summary, multiple novel variants were found to be related to RS in our patient cohort. More importantly, we report a patient carrying biallelic variants in *NXN*, which unambiguously confirms our previous observation that *NXN* can cause recessive RS, i.e. RRS, due to LoF. Finally, our study shows that the genetic and allelic heterogeneity in RS can challenge molecular diagnosis and, in some patients, may require utilizing multiple genomic technologies including aCGH and next-generation sequencing to fully uncover the underlying gene and genetic mechanisms in this Mendelian disorder. For a clinical workflow to establish a molecular diagnosis in RS (Supplemental Figure 1B) we suggest a gene panel for all the known WNT-pathway genes (*DVL1*, *DVL3*, *FZD2*, *NXN*, *ROR2*, and *WNT5A*) followed by ES in unsolved cases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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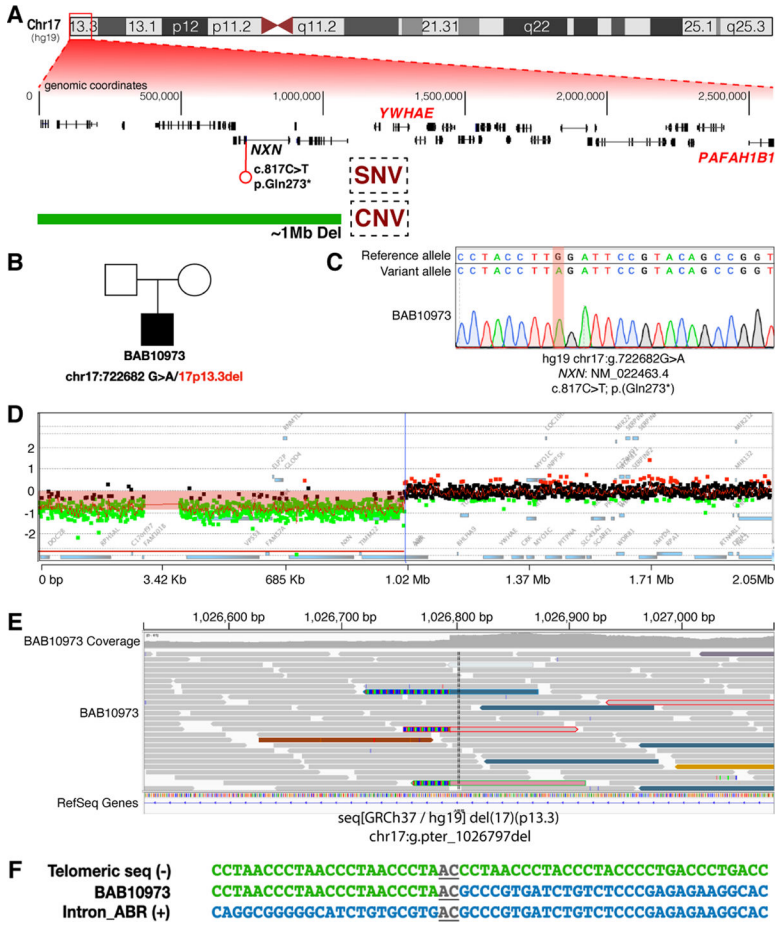

Figure 1. Location of DNA variants affecting *NXN* resulting in RRS

A: Below the chr17 ideogram, region p13.3 (red box) is expanded to show all genes in the genomic interval. The variant c.817C>T (red circle) is indicated on the *NXN* gene, which is a nonsense variant followed by a representation of subtelomeric deletion (green rectangle) containing *NXN*. This deletion does not include *PAFAH1B1* or *YWHAE* related to the Miller-Diecker deletion syndrome. **B:** Family pedigree. Patient BAB10973 carries biallelic variants affecting *NXN*. Parental samples are not available for testing. **C:** Sanger sequencing results confirm the nonsense variant affecting *NXN* as detected by ES; **D:** Array CGH result (AMADID#085772) from chr17p reveals a telomeric deletion spanning 1.027 Mb that includes *NXN* (green probes shadowed by a red rectangle); **E:** Integrative Genomics Viewer (IGV) screenshot showing 30x GS Illumina short-reads spanning the breakpoint junction of the telomeric deletion. Grey dotted line indicates that the breakpoint junction maps at chr17:1,026,797. **F:** Sequencing alignment of the breakpoint junction from the telomeric deletion as confirmed by standard Sanger sequencing. The color-matched junction sequence is aligned to the telomeric repeats and the distal genomic references at *ABR*. Strand of alignment (+ or -) is indicated in parenthesis.

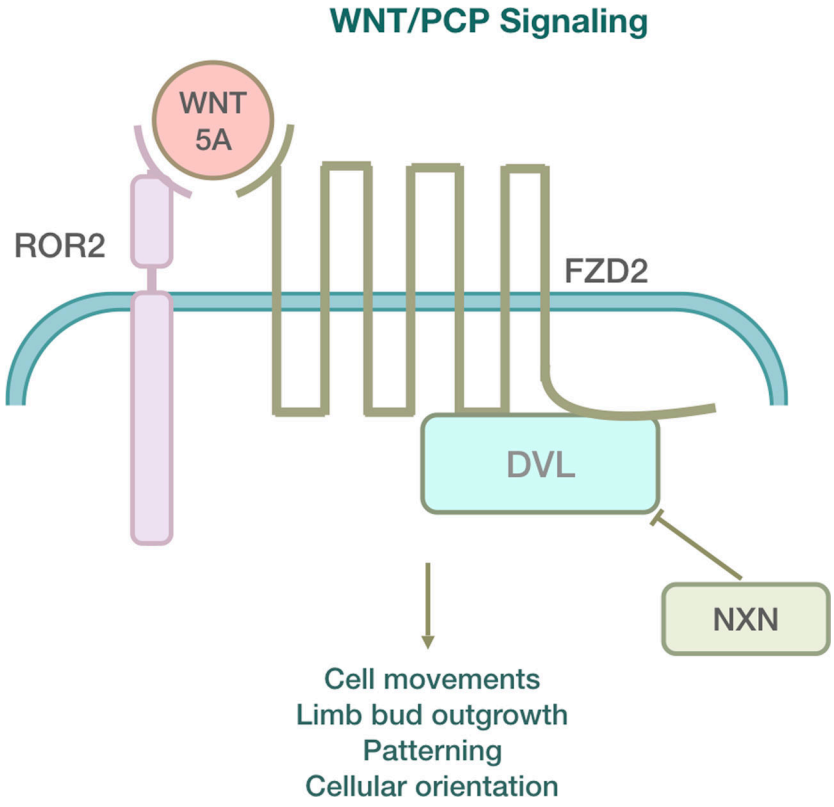

Figure 2. Robinow syndrome-associated genes involved in the WNT/PCP pathway identified in human subjects

The establishment of WNT/PCP signaling is vital for vertebrate development. All Robinow syndrome associated genes result in proteins that play a role in WNT/PCP signaling, therefore, dysregulation of the WNT pathway in humans may be the cause of Robinow syndrome. ROR2 is a co-receptor of FZD2, which binds to extracellular ligand WNT5A, together they can activate the non-canonical WNT signaling, which is associated with planar cell polarity in drosophila and the equivalent convergent-extension movement during gastrulation in vertebrates (Isao Oishi1, 2003; Sokol, 2000; Yamanaka et al., 2002). DVLs are key downstream mediators of the WNT pathway and NXN was found to be a negative regulator of the WNT pathway. This model is adapted and revised from a previous version (White et al., 2018). We removed a potential candidate gene *RAC3* since the clinical phenotype of the patient who has a potentially pathogenic variant in *RAC3* is more consistent with clinically distinct *RAC3*-related neurodevelopmental disorder (Costain et al., 2019; de Curtis, 2019; Hiraide et al., 2019) than with DRS.

Table1:

Summary for variants found in patients diagnosed with RS in our cohort

Individual ID	Gene	Variant Type	Zygosity	Transcript	Variant	Effect	Frequency (gnomAD)	ACMG classification
BAB10151	*DVL1*	−1 fs	Het	NM_004421.2	c.1556del	p.Gly519Aspfs*130	0	Pathogenic
BAB9136	*ROR2*	Missense	Compound Het	NM_004560.3	c.899G>T	p.Cys300Phe	0	Likely pathogenic
		−1 fs		NM_004560.3	c.990del	p.Thr331Profs*114	0	Pathogenic
BAB14232	*ROR2*	Missense	Compound Het	NM_004560.3	c.904C>T	p.Arg302Cys	0.000004455	Likely pathogenic
		Missense		NM_004560.3	c.1970G>A	p.Arg657His	0.00001415	Likely pathogenic
BAB10973	*NXN* + 12 genes	Deletion	Compound Het	NA	seq[GRCh37 / hg19] del(17)(p13.3) chr17:g.pter_1026797del	NA	0	Likely pathogenic
	NXN	Nonsense		NM_022463.4	c.817C>T	p.Gln273*	0	Pathogenic

Het: heterozygous; fs: frameshift; NA: Not Applicable