

ARTICLE

Missense variant in *CCDC22* causes X-linked recessive intellectual disability with features of Ritscher-Schinzel/3C syndrome

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Ritscher-Schinzel syndrome (RSS)/3C (cranio-cerebro-cardiac) syndrome (OMIM#220210) is a rare and clinically heterogeneous developmental disorder characterized by intellectual disability, cerebellar brain malformations, congenital heart defects, and craniofacial abnormalities. A recent study of a Canadian cohort identified homozygous sequence variants in the *KIAA0196* gene, which encodes the WASH complex subunit strumpellin, as a cause for a form of RSS/3C syndrome. We have searched for genetic causes of a phenotype similar to RSS/3C syndrome in an Austrian family with two affected sons. To search for disease-causing variants, whole-exome sequencing (WES) was performed on samples from two affected male children and their parents. Before WES, CGH array comparative genomic hybridization was applied. Validation of WES and segregation studies was done using routine Sanger sequencing. Exome sequencing detected a missense variant (c.1670A > G; p.(Tyr557Cys)) in exon 15 of the *CCDC22* gene, which maps to chromosome Xp11.23. Western blots of immortalized lymphoblastoid cell lines (LCLs) from the affected individual showed decreased expression of *CCDC22* and an increased expression of WASH1 but a normal expression of strumpellin and *FAM21* in the patients cells. We identified a variant in *CCDC22* gene as the cause of an X-linked phenotype similar to RSS/3C syndrome in the family described here. A hypomorphic variant in *CCDC22* was previously reported in association with a familial case of syndromic X-linked intellectual disability, which shows phenotypic overlap with RSS/3C syndrome. Thus, different inactivating variants affecting *CCDC22* are associated with a phenotype similar to RSS/3C syndrome.

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INTRODUCTION

Ritscher-Schinzel syndrome/3C (cranio-cerebro-cardiac) (RSS/3C) syndrome is a rare and presumably autosomal recessive intellectual disability (ID) syndrome with a specific pattern of malformations of the brain and heart as well as minor anomalies of the face and the hands and feet. Approximately 80% of RSS/3C syndrome patients show cardiac defects, which can include septal defects, tetralogy of Fallot, double outlet right ventricle, hypoplastic left heart, aortic stenosis, pulmonic stenosis, and other valvular anomalies. Many patients show Dandy–Walker malformation, cerebellar vermis hypoplasia, posterior fossa cysts, and ventricular dilatation.¹ The facial dysmorphism of RSS/3C syndrome is characterized by a prominent forehead, prominent occiput, downslanting palpebral fissures, depressed nasal bridge, low-set ears, and micrognathia. The phenotypic manifestations of RSS/3C syndrome can vary and cardiac and cerebellar manifestations are not always present. Therefore the pattern of craniofacial dysmorphic features is crucial in ascertaining the diagnosis.^{2,3} A recent study of a cohort of affected individuals of the First Nation community in northern Manitoba, Canada, an isolate, identified homozygous sequence variants affecting

the *KIAA0196* gene, which encodes the WASH (Wiskott–Aldrich Syndrome Protein and SCAR Homolog) complex protein strumpellin as causal to a form of RSS/3C syndrome.² Here we present a study of an Austrian family with two affected male children. To elucidate the molecular basis of their phenotype we applied NGS-based whole-exome sequencing (WES). In this report, we show that a missense variant in *CCDC22* is associated with a form of X-linked ID with features of RSS/3C syndrome. Review of literature about *CCDC22* (coiled-coil domain-containing protein 22) function and its molecular interactions points to a likely role of this protein both in the NF- κ B signaling, and in the function of WASH complex, which is involved in actin polymerization and multiple endosomal transport processes.

MATERIALS AND METHODS

CGH array screening

In proband 1, array-CGH was performed with genomic DNA extracted from peripheral blood on an Agilent 60k Microarray (Agilent Technologies, Santa Clara, CA, USA). Slides were scanned using the Agilent microarray scanner G2505B and images were analyzed using Agilent DNA Analytics software

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4.0.76. The analysis with CGH analytics software was performed with the statistical algorithm ADM-2 with the sensitivity threshold set to 6.0. At least three consecutive oligos with a log ratio of 0.29 had to be aberrant to be called by the software.

Whole-exome sequencing

WES was done on DNA samples of the Austrian family (probands 1, 2 and their parents). Each DNA sample was captured using Agilent SureSelect Human All Exon Kit V2 and sequencing of 100-bp paired end reads was carried out on Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA, USA). Sequence reads were aligned to the haploid human reference genome (hg 19) using Novoalign (Novocraft Technologies, Selangor, Malaysia). Single-nucleotide variants (SNVs) and short insertions and deletions (indels) were called using SAMtools.⁴ All detected variants were annotated with Annovar.⁵ Filtering of common variants present in the Exome Variant Server (EVS), dbSNP132 and the 1000 Genomes database, technical artifacts of sequencing, low-quality data ($Q < 60$), and variants seen in more than three control exomes in our internal data set was performed using a set of Perl scripts. Finally, to predict pathogenic potential of missense variants we used PolyPhen2 and Variant Taster. Only functionally relevant variants predicted to affect protein sequence, that is, coding non-synonymous SNVs, small indels, and splice site SNVs, were considered. Variants were visualized with use of integrative genomics viewer (IGV).⁶ Possible pathogenic variants were then validated with standard Sanger sequencing in all available family members and the CCDC22 variant was deposited in the Human and Clinical Genetics, Leiden University Medical Center – LOVD v.2.0 Build 35 database (URL: <http://grenada.lumc.nl>).

Sanger sequencing

Presence of the variant in *CCDC22* was confirmed with Sanger sequencing, which was done with dye terminator chemistry (ABI Prism BigDye, Life Technologies GmbH, Darmstadt, Germany) and run on automated sequencer Applied Biosystems (Life Technologies GmbH) Prism 3700 DNA Analyzer as previously described.⁷ Sequences of the primers used for exon 15 amplification were:

CCDC22_ex15_F: 5'-ATGAAGCTAGTGGGGTGGTG-3'
CCDC22_ex15_R: 5'-CCACAGCCTCTGTACAGCATA-3'

Western blots

Whole cell lysates of patient and control lymphoblastoid cell lines (LCLs) were resolved by electrophoresis in SDS-polyacrylamide gels and transferred onto Optitran BA-S 85 membrane (GE Healthcare, Fairfield, CT, USA). For western blot analysis, membranes were probed with the antibodies:

anti-GAPDH AM4300 (Ambion, Life Technologies GmbH) 1:10 000; anti-CCDC22 HPA000888 (Sigma-Aldrich GmbH, Seelze, Germany) 1:500; anti-Strumpellin sc-87442 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 1:200; anti-FAM21 sc-137993 (Santa Cruz Biotechnology, Inc.) 1:100; and anti-WASH1 SAB4200372 (Sigma-Aldrich GmbH) 1:500.

Transient transfection and immunofluorescence

HeLa cells were grown on a 24-well plate on glass slides in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum, 100 U/ml penicillin, 100 g/ml streptomycin, and 2 mM glutamine (all reagents from Sigma-Aldrich GmbH) at 37 °C with 5% CO₂. Cells were transiently transfected with pCMV6-Flag-CCDC22 vectors (OriGene, Rockville, MD, USA) (WT, T17A, Y557C), using 4 μl polyethylenimine and 0.4 μg DNA per well. After 24 h, cells were serum-starved in MEM supplemented with 0.2% BSA for 4 h and stimulated with 500 ng/ml EGF-Rh (Invitrogen, Life Technologies GmbH) for 20 min. Following cargo uptake, cells were fixed with 3% paraformaldehyde in PBS for 12 min and permeabilized for 10 min with PBS containing 0.1% w/v saponin, 0.2% w/v gelatin, and 5 mg/ml BSA. Cells were further incubated with EEA1 (BD Transduction Laboratories, San Jose, CA, USA) and Flag (Sigma-Aldrich GmbH) primary monoclonal antibodies. After washing with PBS, cells were incubated with Alexa-labeled secondary antibodies for 30 min. Cells were washed and mounted with Mowiol (Sigma-Aldrich GmbH). Images were acquired with Leica TCS SP2 microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) equipped with AOBS using ×63/1.4 NA oil immersion objective. The presented microscopy images were assembled using Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA, USA).

RESULTS

The affected 8- and 15-year-old brothers are offspring of non-consanguineous healthy parents of Austrian origin. They were born with normal measurements but they developed postnatal growth delay (−3.7 SD in patient 1 and −2.1 SD in patient 2), and a relative large head circumference (occipitofrontal head circumference of +1.3 SD and +0.8 SD, see Table 1). After birth, large anterior fontanelles were documented in both and cryptorchidism was noted in the older one. In addition, both had Dandy–Walker malformations and ventricular septal defects (VSD) for which corrective surgery was performed. Muscular hypotonia and psychomotor delay were noted in both from early infancy. Independent walking was achieved at ages of 21 and 22 months, respectively. At current age they are able to

Table 1 Summary of clinical findings in patients with *CCDC22* variants and *KIAA0196* variants

	Patient 1	Patient 2	Voineagu <i>et al</i> ⁹	Elliott <i>et al</i> ²
Variants	<i>CCDC22</i> variant c.1670A>G	<i>CCDC22</i> variant c.1670A>G	<i>CCDC22</i> variant c.219A>G	Sequence variants of the <i>KIAA0196</i> gene
Intellectual disability	+	+	+	+
Postnatal growth retardation	+ (−3.7 SD)	+ (−2.1 SD)	n.d.	−
Cerebellar malformations	Dandy–Walker malformation	Dandy–Walker malformation	Dandy–Walker malformation, defects of posterior fossa, cerebellar hypoplasia	Structural posterior fossa malformations
Cardiac defects	VSD	VSD	ASD, VSD, dextrocardia	VSD, ASD, Tetralogy of Fallot
<i>Craniofacial</i>				
Head circumference	Relative macrocephaly (OFC +1.3 SD)	Relative macrocephaly (OFC +0.8 SD)	n.d.	Macrocephaly
Hypertelorism	+	+	+	+
Large anterior fontanelle	+	+	n.d.	+
Limb anomalies	Camptodactyly, reduced distal creases, overriding toes	Camptodactyly, reduced distal creases, overriding toes	Absent distal creases, overriding toes	Absent distal creases, syndactyly, brachydactyly, camptodactyly
Other	Cryptorchidism, glaucoma	−	Cryptorchidism, aplasia cutis, hypodontia	Coloboma

Abbreviations: ASD, atrial septal defect; OFC, occipitofrontal head circumference; VSD, ventricular septal defect; n.d., not documented.

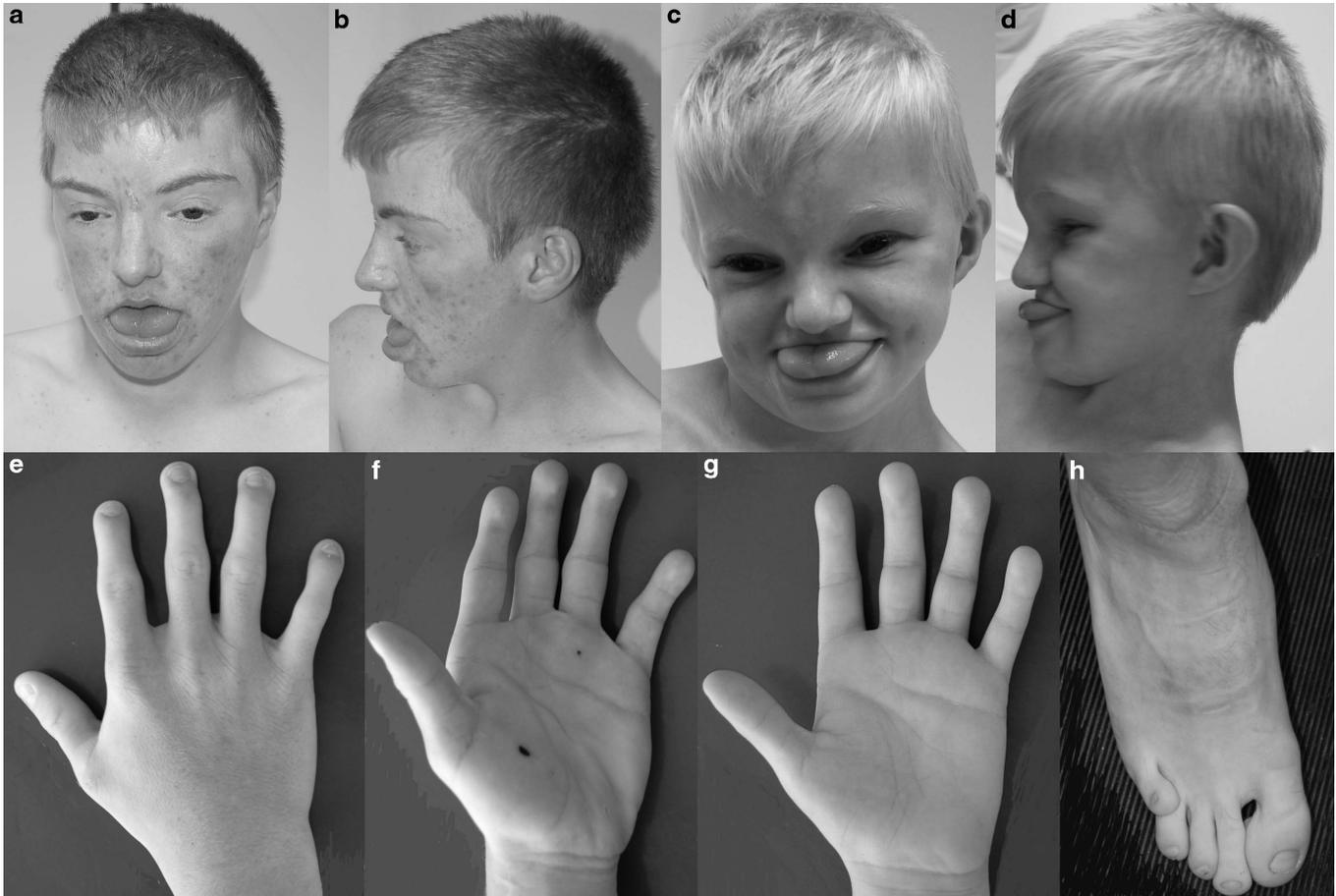


Figure 1 Facial photographs of patient 1 (a, b) and patient 2 (c, d) showing upslanting palpebral fissures, wide-set eyes, short philtrum, protruding tongue, and a broad neck. Hand anomalies of patient 1 (e, f) and patient 2 (g). Note camptodactyly, reduced distal creases of the second to fifth fingers, clinodactyly of the fifth fingers, and hypoplastic finger nails of the second to fifth fingers. Foot of patient 1 (h) showing a broad hallux and an overriding fifth toe.

communicate in simple, dysgrammatic phrases with poor articulation, while receptive speech is well preserved. Their common facial signs included a broad forehead, upslanting palpebral fissures, wide-set eyes, a short philtrum, and a broad neck with a low posterior hair line (Figures 1a–d). In addition, they had a large, protruding tongue. Their fingers showed camptodactyly and reduced distal creases of the second to fifth fingers, clinodactyly of the fifth fingers, fetal finger pads as well as hypoplastic finger nails of the second to fifth fingers (Figures 1e–g). Their halluces were broad and their toes were long with overriding fifth toes (Figure 1h).

We first performed 1.4 M aCGH in proband 1 to test for copy number variations that could potentially account for the observed phenotype. All results of this analysis were normal; therefore we decided to proceed with WES of DNA samples from both probands and their parents. Following sequence alignments, each data set contained >130 000 variant calls and allowed for high-quality calls for over 90% of the human consensus coding sequence. We filtered variant call format files of the four family members by applying homozygous, compound heterozygous, and *de novo* filters in GeneTalk⁸ and viewed variants using IGV browser.⁶ The only biologically relevant variant detected by homozygous filtering was a missense variant located on chromosome chrX:hg19:g.49106004 in exon 15 of the gene *CCDC22* (NM_014008.4) at position c.1670A>G; p.(Tyr557Cys) (Figure 1b). There were no variants annotated at this

position in the exome variant server ((URL: <http://evs.gs.washington.edu/EVS/>) March 2014). The detected variant introduced an amino-acid exchange at the evolutionarily conserved position within a coiled-coil domain of the protein (Figures 2b and c). We performed segregation analysis and showed that the healthy brother and three other unaffected family members (father, maternal brother and maternal sister) did not harbor this variant, whereas the unaffected mother was a heterozygous carrier (Figure 2a).

To further explore the effect of the *CCDC22* p.(Tyr557Cys) variant, we measured the expression of *CCDC22* and *WASH* complex components in the LCLs derived from proband 1, his mother, and a control non-related healthy individual. Western blot analysis revealed a 50% decreased expression of *CCDC22* in the patient cells as compared with those of the healthy mother and an unrelated control (Figure 3). Interestingly, we also detected an increased level of *WASH1* expression in those cells, while the expression levels of strumpellin and *FAM21* were not changed. Thus, p.(Tyr557Cys) variant negatively impacts *CCDC22* protein stability and is associated with increased abundance of *WASH1* protein. Transient expression of wild-type *CCDC22* in HeLa cells resulted in a punctate distribution corresponding to vesicles (Figure 4). No clear overlap with the early endosome marker *EEA1* or endocytosed *EGF* was observed. Expression of *CCDC22-T17A*, and *CCDC22-Y557C* revealed no significant differences in the subcellular distribution.

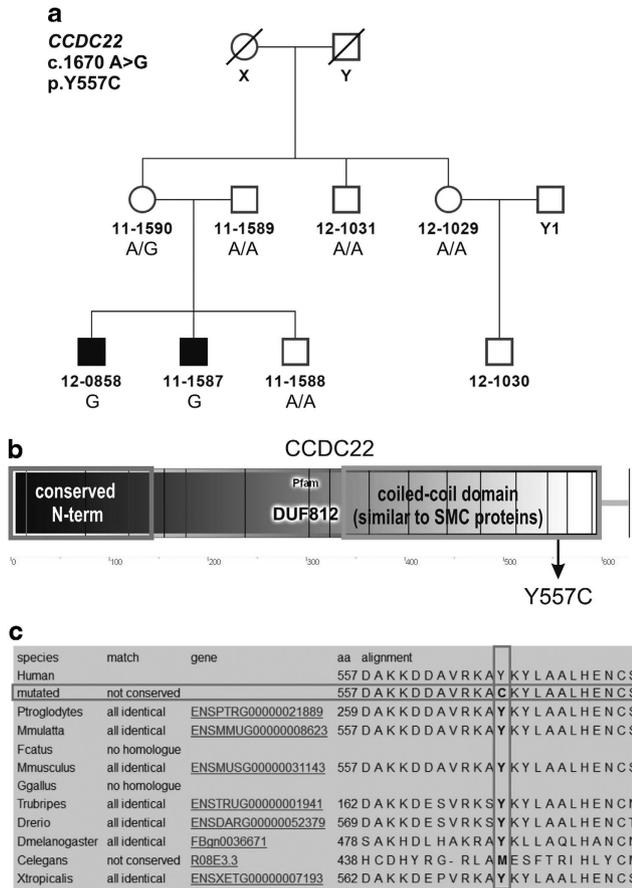


Figure 2 A pedigree of the family affected by the CCDC22 variant. (a) Occurrence of the c.1670A>G variant in the family affected with RSS/3C syndrome. The CCDC22 genotypes are annotated below each tested family member. (b) Schematic representation of the CCDC22 protein structure with annotated functional domains and the location of the p.Y557C variant within the coiled-coil domain of the protein. (c) Evolutionary conservation of the mutated amino-acid residue in the CCDC22.

DISCUSSION

The presented study documents a variant in *CCDC22* gene being a cause of syndromic form of ID classified by us as similar to RSS/3C syndrome. A different *CCDC22* variant has been recently shown to be a cause of X-linked ID (XLID) and involvement of *CCDC22* in ID etiology awaited independent confirmation.^{9,10}

CCDC22 is a highly conserved and broadly expressed protein that has been shown to interact through its N-terminal part with COMMD proteins.¹¹ COMMD proteins take part in multiple processes including regulation of NF- κ B signaling, copper export from the liver, HIV infection, and sodium transport.¹² The *CCDC22* deficiency or a hypomorphic variant in this gene that impairs the interaction with COMMD proteins has been shown to lead to blunted activation of NF- κ B.¹¹ *CCDC22* was also shown to interact with copines,¹³ a family of calcium-dependent membrane-binding proteins, with Nance–Horan syndrome protein (NHS),¹⁴ which is an important regulator of actin cytoskeleton dynamics,¹⁵ as well as with FAM21,¹⁶ a crucial component of the WASH complex that is composed of four additional core proteins: SWIP, strumpellin, *CCDC53*, and *WASH1*. The WASH complex controls the polymerization of actin at the surface of endosomes through the activation of the Arp2/3 complex, a major actin nucleator.¹⁷ WASH-dependent actin polymerization promotes scission of transport intermediates of the different endosomal routes and affects recycling,¹⁷ degradation,¹⁸ and retrograde pathways.^{19,20} Inactivating variants in the gene encoding the WASH subunit strumpellin has been recently shown to cause a form of RSS/3C syndrome.² Variant in another component of the WASH complex, SWIP, was shown to be causal to non-syndromic ID.²¹ Thus, variants in WASH complex components can result in various forms of ID.

The splice site variant NM_014008.4; c.219A>G/p.(Thr17Ala) in the *CCDC22* gene was previously shown to be a cause of syndromic XLID (OMIM 300859) in a large family with six affected males.⁹ This study did not specifically describe the phenotype of the affected individuals as RSS/3C syndrome; however, it provided clinical data according to which the phenotype overlaps with RSS/3C syndrome and includes: presence of ID, Dandy–Walker malformation, cerebellar

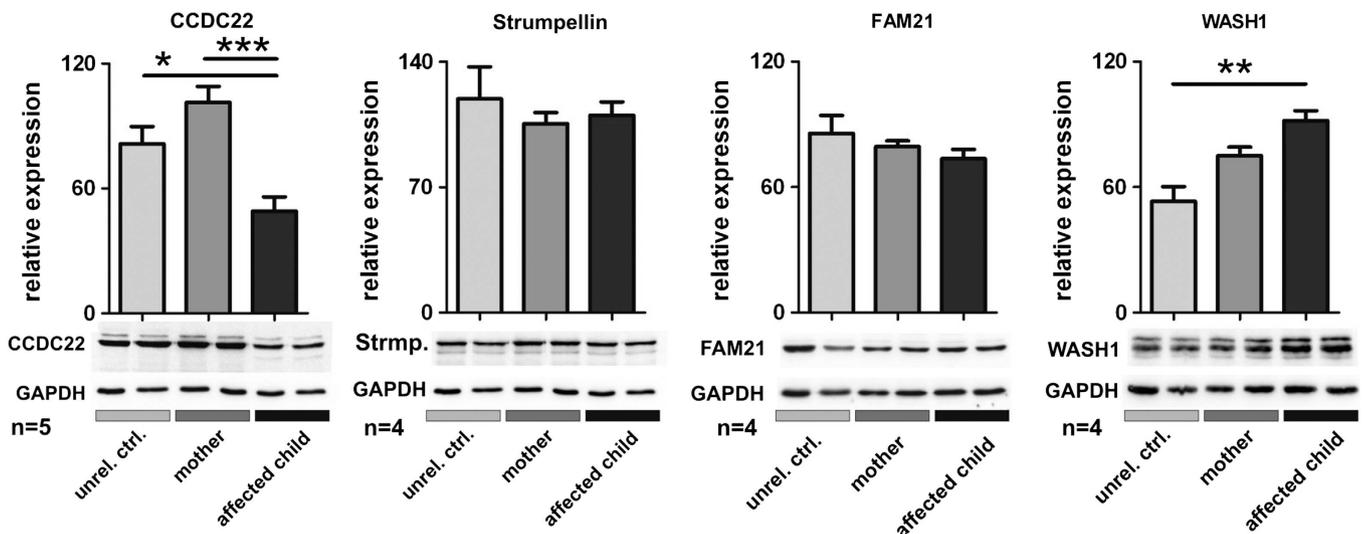


Figure 3 Western blot-based quantification of the *CCDC22*, strumpellin, *Fam21*, and *Wash1* expression in the lymphoblastoid cell lines cells from affected patient, healthy mother, and non-related healthy control. Note a decreased expression of *CCDC22* and increased expression of *WASH1* in the patient cells. Statistical significance – *t*-test, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

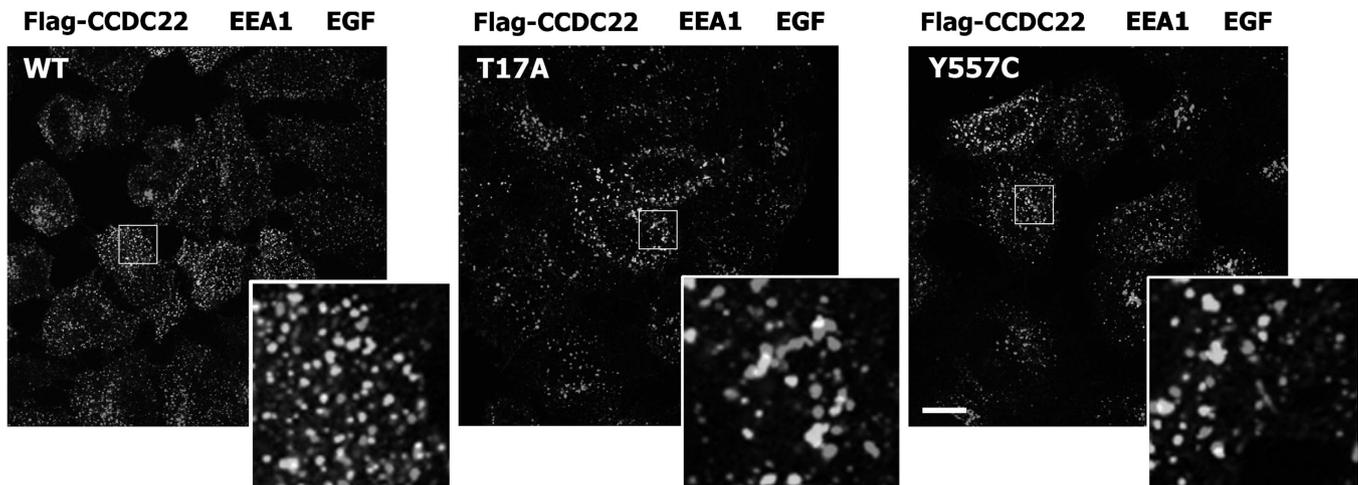


Figure 4 Subcellular distribution of CCDC22 variants associated with RSS/3C spectrum disorders upon EGF uptake. HeLa cells were transfected either with WT, T17A, or Y557C Flag-tagged CCDC22 vectors and subjected to EGF uptake. Cells were immunostained for EEA1 (in red), Flag (in green), and EGF fluorescence is presented in blue. Scale bar = 20 μ m. The full colour version of this figure is available at *European Journal of Human Genetics* online.

hypoplasia, cardiac defects (atrial septum defect, patent ductus arteriosus, VSD, hypoplastic right pulmonary artery), specific facial features (hypertelorism, beaked nose and broad nasal tip, ear abnormalities, high arched palate), as well as various limb anomalies (see Table 1). In addition, two individuals were reported to show signs of ectodermal dysplasia, namely aplasia cutis and dental abnormalities.¹¹ A series of other variants in *CCDC22* was recently described in patients with non-syndromic XLID.¹¹ These included the following missense variants: p.(Thr30Ala), p.(Arg128Gln), p.(Glu238Lys), p.(Arg321Trp), all localized in the N-terminal and mid part of the protein. Unfortunately no clinical data of the individuals affected with these variants were provided. Whereas the previously mentioned variant p.(Thr17Ala) affected the interaction with COMMD1, none of the other variants disturbed this binding when tested in LCLs or after expression in HEK293 cells.¹¹ Nevertheless, this study concluded that CCDC22 is required for the ubiquitination and subsequent turnover of κ B proteins, which is dependent on its interactions with COMMD proteins. As an additional evidence for altered NF- κ B signaling, the signs of ectodermal dysplasia observed in a minority of patients were presented. Ectodermal dysplasia is known to result from of κ B- α variants that alter its degradation or variants in the ectodysplasin pathway, a TNF-related signaling cascade that is involved in ectodermal development.¹¹

The two patients described here show a broad phenotypic overlap with RSS/3C syndrome described in the literature. In addition to ID, both displayed Dandy–Walker malformation, VSD, hand and foot anomalies (camptodactyly, clinodactyly, reduced distal creases of fingers, hypoplastic finger nails, and overriding toes), and facial dysmorphism. These two probands show a hemizygous variant in the *CCDC22* gene that is localized to the C-terminally located coiled-coil domain. Although this variant can potentially have different effects on the protein function and partner protein interactions than the N-terminally localized p.(Thr17Ala) variant, the observed phenotypic features in both families are strikingly similar and consistent with the diagnosis of RSS/3C syndrome (Table 1). However, it should be noted that the short stature and relative large head circumference are not mentioned for the individuals carrying the *CCDC22* variant p.(Thr17Ala).

In the light of the recent report about strumpellin intronic variants (c.3335 + 2T > A, c.3335 + 4C > A and c.3335 + 8A > G) being causal

to a form of RSS/3C syndrome,² it is interesting to note that CCDC22 was shown to interact with FAM21, a subunit of the WASH complex.¹⁶ Apart from ID, phenotypic overlap in both patient groups consists of the same pattern of cerebellar and cardiac as well as hand and foot anomalies (Table 1). However, facial dysmorphisms in both groups are different. Whereas individuals with homozygous *KIAA0196* sequence variants show macrocephaly, down slanting palpebral fissures, and low set ears, the patients reported here present with different facial features, for example, occipitofrontal circumference in the upper normal range, upslanting palpebral fissures, and normal appearing ears (Figures 1a–d). Furthermore, the affected individuals of the family reported here display postnatal short stature in contrast to normal growth in the affected families carrying the homozygous sequence variants of the *KIAA0196* gene. Nevertheless, it seems likely that variants in CCDC22 affect the same molecular machinery as variants in strumpellin. Our observation that WASH1 expression level is increased in the CCDC22-mutant LCL cells supports the notion of the possible link between CCDC22 and WASH complex function.

One remaining question is how two missense variants (p.(Thr17Ala) and p.(Tyr557Cys)) in two very distant regions of the CCDC22 protein lead to such closely related phenotypes. As indicated by our immunoblot analysis, CCDC22-Y557C shows significantly reduced protein stability in LCLs from patients, which is likely caused by a premature or accelerated degradation of mutant protein, provoked by aberrations of its conformation.²² Likewise, the c.219 A > G change leading to the very mild exchange p.(Thr17Ala) entails a splice defect, which is not unlikely to also lead to reduced levels of the wild-type protein. Unfortunately, no protein expression was investigated in this previous study. Another finding supporting this hypothesis is the comparable vesicular pattern of wild-type and mutated CCDC22 after transient expression, which, however, does not preclude functional changes independent of intracellular sorting events.

In conclusion, we show that *CCDC22* variants, leading to at least partial loss of the protein, are associated with ID, cerebellar anomalies, cardiac defects, facial dysmorphism, as well as hand and foot anomalies. Our findings widen the phenotypic as well as the genotypic spectra of syndromic XLID caused by variants in *CCDC22*. Together with previous reports this suggests that the clinical spectrum

associated with *CCDC22* variants can range from syndromic manifestations similar to RSS/3C syndrome to non-syndromic ID. Given the marked clinical variability of RSS/3C syndrome and the heterogeneous etiology, we suggest to collectively call this group of conditions WASHopathies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MK: analyzed data, wrote the manuscript; PK: analyzed data, provided bioinformatics support; JH: coordinated NGS; AH, CS: performed the experiments; MM: provided molecular expertise, reviewed the manuscript; KM: conducted western blots; DE: reviewed the manuscript; KK: reviewed the manuscript; GG and SU: studied the patients, collected the samples, helped in the clinical work; BP, GG, and SU: contacted the family, collected clinical material, and reviewed the manuscript; UK: reviewed the manuscript; PNR: provided bioinformatics support, SM: supervised the experimental work, provided financial support for the research and critically revised the manuscript; DH: analyzed the clinical data and wrote the manuscript.

ETHICS APPROVAL

Patient consent was obtained for this study. Ethics approval was granted by the Institutional Review Board.

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