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Identification of a novel candidate gene for non-syndromic autosomal recessive intellectual disability: the WASH complex member SWIP

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High-throughput sequencing has greatly facilitated the elucidation of genetic disorders, but compared with X-linked and autosomal dominant diseases, the search for genetic defects underlying autosomal recessive diseases still lags behind. In a large consanguineous family with autosomal recessive intellectual disability (ARID), we have combined homozygosity mapping, targeted exon enrichment and high-throughput sequencing to identify the underlying gene defect. After appropriate single-nucleotide polymorphism filtering, only two molecular changes remained, including a non-synonymous sequence change in the SWIP [Strumpellin and WASH (Wiskott-Aldrich syndrome protein and scar homolog)-interacting protein] gene, a member of the recently discovered WASH complex, which is involved in actin polymerization and multiple endosomal transport processes. Based on high pathogenicity and evolutionary conservation scores as well as functional considerations, this gene defect was considered as causative for ID in this family. In line with this assumption, we could show that this mutation leads to significantly reduced SWIP levels and to destabilization of the entire WASH complex. Thus, our findings suggest that SWIP is a novel gene for ARID.

INTRODUCTION

Developmental delay/intellectual disability (DD/ID) is the most common reason for referral to genetic services, and with a prevalence of 1–3%, it is one of the largest unresolved problems of health care worldwide (1). It is particularly frequent in countries where parental consanguinity is common (2), which points to the involvement of autosomal recessive gene defects. Compared with X-linked forms of DD/ID, which have received much attention during the past decade, the search for gene defects underlying autosomal recessive forms of DD/ID is still in its infancy. The reason for this is that most of this research takes place in industrialized countries where parental consanguinity and large families are rare.

To date, only seven gene defects have been unequivocally linked to non-syndromic autosomal recessive ID (ARID), i.e. PRSS12, CC2D1A and CRBN (3–5) and, more recently, GRIK2, TUSC3, TRAPPC9 and TECR (6–12). ID is a very heterogeneous disorder, as illustrated by the fact that more than 30 genes for non-syndromic ID have been identified on the X chromosome, which is thought to contain only ∼4% of the human genes. Therefore, the few known ARID genes are likely to represent only the tip of the iceberg (13–14).

Recently, efficient novel genomic enrichment techniques and high-throughput sequencing have greatly facilitated

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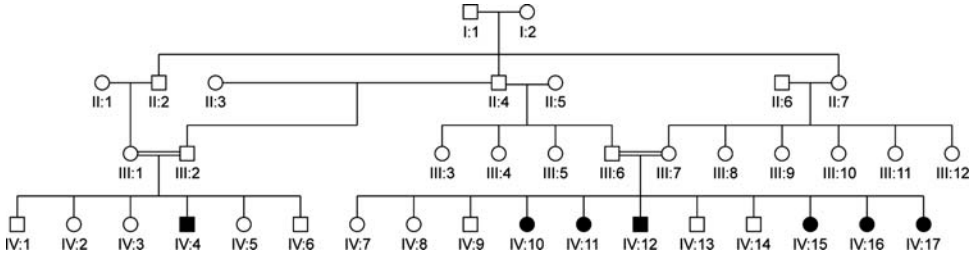

Figure 1. Pedigree of the family. All patients (dark symbols) have moderate to severe ID.

mutation screening. We applied these approaches to a large consanguineous family with ARID.

Figure 1 shows the pedigree of this family. In its two branches, there are seven affected patients, five females and two males, all with non-syndromic ARID.

All affected children had moderate to severe ID/DD (IQ 35–50). They had severe learning impairment, poor language skills and poor adaptive skills. Age of walking was normal, though other (especially fine) motor development was severely delayed. They were of short stature (third centile), with normal head circumference. Non-genetic causes for mental retardation could be excluded, and there were no dysmorphic features. Whereas ID was the only clinical abnormality in six of the seven affected children, subject IV:4 also showed signs of spasticity. Magnetic resonance imaging (MRI) was normal, but in the absence of perinatal monitoring, a history of perinatal hypoxia could not be ruled out.

By combining homozygosity mapping, targeted exon enrichment and high-throughput sequencing, we were able to identify a mutation in kiaa1033, encoding SWIP [Strumpellin and WASH (Wiskott-Aldrich syndrome protein and Scar Homolog)-Interacting Protein]. We provide the first evidence that destabilization of the WASH complex through a missense mutation in one of its subunits leads to a severe cognitive disorder.

RESULTS

Linkage analysis on four affected and six unaffected subjects yielded a single homozygous interval defined by flanking heterozygous single-nucleotide polymorphism (SNP) markers at positions 102615255 (rs7312283) and 115621170 (rs11068147) (human genome version 18) on chromosome 12q23-24, in a region not previously implicated in ARID (logarithm of odds score 3.5). These results were refined by genotyping a panel of 19 microsatellite markers in 11 of 12 siblings and both parents of the larger family branch. This resulted in an interval of 12.5 Mb between positions 103 065 571 and 115 621 170 (data not shown).

This region contains 159 genes (including *ORFs*, *Genedistiller*). In subject IV:12, high-throughput sequencing of all coding sequences in the 12 Mb target region yielded 49 homozygous variants. Forty-six of those were evaluated as presumably neutral polymorphisms by comparison with dbSNP130 (http://www.ncbi.nlm.nih.gov/projects/SNP/), the genomic sequence of 185 individuals available through the 1000-genome project (15) and 200 recently published exomes from Danish individuals (16). The remaining three variants were annotated using RefSeq as basis (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/database/refGene.txt.gz, July 2010). Subsequent screening for non-synonymous mutations and changes involving canonical splice sites revealed two potentially disease-causing mutations.

In exon 29 of *kiaa1033*, we identified a homozygous, probably disease-causing missense mutation, c.3056C > G. This mutation results in a Pro1019Arg exchange in SWIP, a member of the WASH complex. Another homozygous c.1085A > T (p.His362Leu) variant was observed in exon 5 of *lhx5*, an LIM homeobox gene. Both changes were validated by Sanger sequencing, and co-segregation with ID was confirmed by re-analysis of the family.

Further evaluation with PhyloP (44vertebrates) (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/database/phyloP44wayAll.txt.gz) yielded high base conservation scores for both changes. To estimate the pathogenicity of these variants, PMUT (17), SIFT (18), PolyPhen2 (19) and MutationTaster (20) were employed. While the KIAA1033 change was considered as pathogenic throughout, the LHX5 variant yielded ambiguous results (Table 1), which was supported by clinical evidence and functional considerations (see below).

Analysis of the 1000-genome database and 200-Danish-exome data set did not reveal any deleterious mutations in these genes, and this was corroborated by Sanger sequencing of the respective mutated exons in healthy individuals from Oman (331 controls for *kiaa1033*, 365 for *lhx5*). Finally, four unrelated consanguineous Iranian families with ARID and regions of homozygosity overlapping the relevant linkage interval on chromosome 12q23-24 were screened for mutations in these two genes. No additional mutations could be identified.

Lhx5 is a member of the LIM-homeobox genes that encode a family of transcription factors that are highly conserved throughout evolution (21–22). It contains two zinc finger motifs called LIM domains (AA3–125) located N-terminally of the DNA-binding homeodomain (AA180–239) (23). The amino acid exchange at position 362 was located outside the known functional domains of LHX5.

The gene is solely expressed during the embryonic development and plays a role in the anatomic formation of the hippocampus (24). Since inactivation of the Lhx5 gene in mouse results in gross anatomical changes in the hippocampus, we reasoned that pathogenic mutations in humans leading to a severe cognitive disorder would be likely to induce observable anatomical changes in the brain. However, MRI scans of five patients did not reveal structural abnormalities of the hippocampal area or other brain regions (except for a benign midline cyst

Table 1. Sequencing information and pathogenicity predictions for Pro1019Arg in SWIP and His362Leu in LHX5

Mutation	Gene	Support. reads	Allelic percentage	PhyloP (44 vertebrate)	Pmut	SIFT	PolyPhen2 (HumVar-trained)	Mutation taster
g.104078052C > G c. 3056C > G p. P1019R	KIAA1033 (NM_015275)	21	100	5.37	PATHOLOGICAL	Not scored	Probably damaging	Disease causing
g.112385502T > A c.1085A > T p.H362L	LHX5 (NM_022363)	39	81	4.09	PATHOLOGICAL	TOLERATED	Benign	Disease causing

in subject IV:11), thereby providing another argument against the pathogenicity of the Lhx5 variant in this family. Therefore, subsequent studies to elucidate the pathogenesis of ID in this family focused on the apparently pathogenic mutation of the SWIP gene.

SWIP is a large ubiquitously expressed protein of 1173 amino acids, which has no identifiable domain. However, it has recently been described as a subunit of the WASH complex (25–26). 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. WASH-dependent actin polymerization promotes scission of transport intermediates of the different endosomal routes and affects recycling (25), degradation (27) and retrograde pathways (28,29).

The stable assembly of the WASH complex involves a core of five subunits, namely SWIP, FAM21, strumpellin, Ccdc53 and WASH, and the recruitment of the heterodimer of Capping Protein (25,30). These studies reported that the stability of WASH and Ccdc53 subunits depends on all the other subunits, whereas the stability of a subcomplex formed by SWIP, FAM21 and strumpellin does not depend on WASH and Ccdc53.

To examine the effect of the potentially pathogenic mutation of SWIP, we isolated stable cell lines of 3T3 cells expressing either wild-type SWIP or Pro1019Arg-mutated SWIP. These two lines were generated by homologous recombination at a defined locus in order to ensure similar expression of the two transgenes. The exogenous proteins encoded by the transgenes are tagged with the protein C (PC) epitope.

The exogenous wild-type SWIP was clearly detected by PC western blot, but was at the limit of detection, when the PRO1019ARG mutation was present (Fig. 2). This result suggests that the mutation PRO1019ARG destabilizes the SWIP. However, WASH was present at similar levels in both cases, indicating that expression of the mutant SWIP has no dominant negative effect on the assembly or maintenance of the WASH complex in the presence of endogenous wild-type SWIP. This finding is in line with the recessive mode of inheritance of the condition.

We established lymphoblastoid cell lines from patients, healthy relatives and unrelated healthy subjects. Protein extracts from the cell lines were analyzed by western blot using the two antibodies available to us for the WASH complex, our home-made WASH antibody (25) and a commercially available strumpellin antibody. The affected patients, carrying the homozygous Pro1019Arg mutation of the SWIP, exhibited reduced levels in both strumpellin and WASH compared with healthy relatives. Healthy relatives had similar levels of strumpellin and WASH, independent of their carrier status (Fig. 3). These results indicate that the Pro1019Arg mutation of SWIP impairs the stability of the WASH complex, when mutant SWIP is the sole source of this subunit.

Thus, we were able to show that the substitution of the highly conserved proline at position 1019 of SWIP leads to markedly reduced concentrations of this protein and the WASH complex it is embedded into.

DISCUSSION

By combining homozygosity mapping and high-throughput sequencing in a large consanguineous family, we were able to identify a new candidate gene, SWIP, that is involved in non-syndromic ARID. This gene encodes a subunit of the WASH complex. WASH has recently been shown to activate the Arp2/3 complex, which is known to generate branched actin networks (31). The WASH complex is located at the surface of endosomes (25,27,28) and it promotes membrane scission of transport intermediates through the branched actin network it induces (25). This newly discovered complex has already been implicated in numerous endosomal transport pathways, namely the recycling pathway (25), the degradation pathway (27) and the retrograde pathway (28–29). These trafficking pathways are especially important in neurons, where cargoes have to travel long distances. Alterations in these pathways are at the origin of several forms of neuropathies (32).

Importantly, we have been able to demonstrate that the mutation of SWIP found in patients with ID has a deleterious effect on the stability of SWIP and the entire WASH complex. In stable multiprotein complexes, like WASH or the analogous Wasp family, verprolin homology domain-containing protein complex, which plays a similar role in activating Arp2/3 in lamellipodia, subunits usually depend on each other for their stability (33). By depleting SWIP expression, it was shown that SWIP is required for the stability of all the other core subunits of the WASH complex (30). Here, we found the same effect in lymphoblastoid cell lines derived from the patients, indicating that the single point mutation Pro1019Arg is able to exert the same effect as the absence of SWIP. In line with the autosomal recessive transmission in our family of ID patients, we have found that the expression of the mutant SWIP subunit does not impair the stability of the WASH complex in a dominant-negative way.

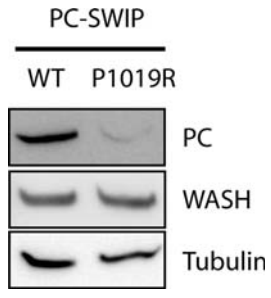

Figure 2. The mutation PRO1019ARG destabilizes SWIP. Stable 3T3 cell lines were established, expressing either PC-tagged wild-type (WT) SWIP or Pro1019Arg SWIP from the same locus to ensure similar expression. Cell extracts were analyzed by western blot. The level of PRO1019ARG SWIP is strongly reduced compared with WT SWIP. The expression of PRO1019ARG has no effect on the level of WASH complex.

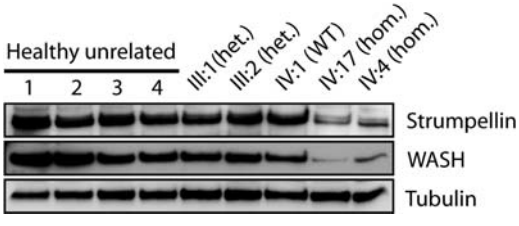

Figure 3. The PRO1019ARG mutation of SWIP destabilizes the WASH complex. Extracts prepared from different lymphoblastoid cell lines were analyzed by western blot with the indicated antibodies. Strumpellin and WASH are subunits of the WASH complex, whereas Tubulin is a loading control. Patients and healthy relatives from the family, whose pedigree is shown in Figure 1, as well as healthy unrelated subjects were analyzed. The homozygous patient IV:17 has a severely reduced amount of WASH and strumpellin, compared with his two parents, III:1 and III:2, which are heterozygous for the PRO1019ARG mutation, and the other controls. A similarly reduced amount of WASH complex is observed in a cousin, patient IV:4, who is also homozygous for the PRO1019ARG mutation of SWIP.

Interestingly, another subunit of the WASH complex has been implicated in a hereditary neurological disease. Mutations in the subunit strumpellin were identified as a rare cause of autosomal dominant hereditary spastic paraplegia, a disease characterized by the degeneration of upper motor neurons with onset from the third decade (34). However, these strumpellin mutations did not lead to destabilization of the WASH complex (30), suggesting that the mutations affect other protein–protein interactions than the ones required for complex assembly or maintenance.

Multiple other ID genes are involved in actin dynamics and vesicle trafficking. STXBP1 (35) and SYP (36) encode proteins that associate with soluble NSF [N-ethylmaleimide-sensitive factor] attachment protein receptor complexes, involved in membrane fusion. GDI1 and CASK regulate the Rab3 GTPase (37–38), important for neurotransmitter release and synaptic plasticity. Several ID genes are associated with the Rho family of GTPases that also play a prominent role in actin dynamics. Examples are OPHN1 that acts as a Rho GTPase-activating protein (39), ARHGEF6 and FGD1, encoding guanine nucleotide exchange factors for Rac1 and Cdc42 (40–42) or the Rac and Cdc42 effectors, PAK3 (40,41,43). Here, it is worth mentioning that WASH has been found to be regulated by Rho in *Drosophila* (44), even though this may not be the case for the human WASH complex (30). Taken together, our studies indicate that mutated SWIP and its deleterious effect on WASH complex assembly leads to severe neuronal malfunction, either through interaction with the Rho pathway or by affecting other endosomal pathways. In view of the compelling evidence for a causative role of SWIP, and the arguments against an involvement of LHX5 in this disorder, we consider it less likely that the mutations found in the two closely linked and therefore co-segregating genes LHX5 and KIAA1033 both contribute to the described phenotype, but at least formally, such a digenic model cannot be excluded.

MATERIALS AND METHODS

Patients

The family was identified and examined in the genetic clinic of the Royal Hospital, Muscat, Oman. MRI scans were performed in five patients (IV:4, IV:10, IV:11, IV:16 and IV:17). Apart from a benign cerebellar midline cyst in subject IV:11, no abnormalities were observed.

After informed consent was obtained from the parents, blood samples were collected from all but one individual (IV:8). DNA was extracted according to a standard procedure. Lymphoblastoid cell lines were established from affected subjects IV:4 and IV:17, and several healthy family members. Whole-genome SNP genotyping was performed using Affymetrix GeneChip® Human 250 K Sty I Array on four of seven patients (IV:10, IV:11, IV:16 and IV: 17), the parents (III:6 and III:7) and four unaffected siblings (IV:7, IV:9, IV:13 and IV:14) following the manufacturer's instructions. For linkage analysis, Allegro (45) was used for parametric multipoint linkage analysis assuming the autosomal recessive mode of inheritance and complete penetrance.

Exon enrichment and high-throughput sequencing

DNA from subject IV:12 was extracted using the standard procedures. Array-based target enrichment of the homozygous region on chromosome 12 with the Agilent SureSelect DNA Capture Array© was used to selectively amplify all 1376 exons plus 50 bp flanking sequences of the 159 genes with a total length of 392 165 bp, following the manufacturer's instructions.

Subsequently, high-throughput sequencing was carried out with the Illumina Genome Analyzer II GAII. Thirty-six base pair single read sequencing of the enriched DNA was performed according to the respective Illumina manual. The total read length encompassed 416 Mb, of which 394 Mb could be mapped to the human reference genome. The total read length in the target region amounts to 23 Mb with a coverage of 99.3%. A mean coverage of 121-fold was reached for the target region.

We aligned the 36 bp single-end reads onto human reference genome (hg18) (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/chromosomes/) using SOAP2.20 (46) with parameter settings –a –D –o. Reads that were unambiguously aligned to target regions were used for variant calling. For variant calling, multiple criteria were used: presence of at

least three non-identical supporting reads, Phred-like quality score >20 and allelic percentage >70.

Generation of 3T3 cell lines stably expressing KIAA1033

The DNA fragment encoding full-length KIAA1033 was amplified by polymerase chain reaction using the cDNA clone IMAGE:8143997 as a template, then cloned into a modified pCDNA5/FRT/V5-His (Invitrogen) vector in which the CMV promoter was replaced by a EF1α/HTLV chimera promoter amplified from pFUSE FC (Invivogen). This vector tags SWIP at its N-terminus with $(His)_6$-PC (HHHHHH-EDQVDPRLIDGK) followed by a tobacco etch virus protease-binding and cleavage site (DYDIPTTEN-LYFQG). The c.3056C > G point mutation was introduced using the Quick Change site-directed mutagenesis kit (Stratagene).

Flp-In NIH3T3 cells (Invitrogen) were grown in Dulbecco's modified eagle medium supplemented with 10% donor calf serum (Invitrogen). They were transfected with Lipofectamine 2000 (Invitrogen). Stable transfectants obtained by homologous recombination at the flp recombination target (FRT) site according to the manufacturer's instructions were selected using 100 μg/ml Hygrogold (Invivogen).

Cell extracts and western blotting

About 5×10^6 cells were washed in phosphate-buffered saline then lysed in 400 μl of RIPA (50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% nonyl phenoxypolyethoxylethanol-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS); pH 7.7) supplemented with Protease Inhibitor Cocktail (1:500, Sigma). Lysate was rocked for 10 min at 4°C then spun at 20 000*g* for 10 min at 4°C. SDS–polyacrylamide gel electrophoresis was performed using 4–8% Tris-acetate gels (47).

Western blots were revealed using horseradish peroxidase coupled secondary antibodies, Supersignal kit (Pierce) and a Fuji LAS-3000 (Fujifilm).

Polyclonal antibodies targeting WASH were described previously (25). Anti-tubulin monoclonal antibody (mAb) (clone E7) was developed by M. Klymkowsky and obtained from Developmental Studies Hybridoma Bank. Strumpellin pAb (C-14) and PC-tag mAb were from Santa Cruz and Roche, respectively.

WEB RESOURCES

http://www.genedistiller.org, January 2011
http://hgdownload.cse.ucsc.edu/goldenPath/hg18/chromosomes, January 2011
http://www.ncbi.nlm.nih.gov/projects/SNP, January 2011
http://hgdownload.cse.ucsc.edu/goldenPath/hg18/database/refGene.txt.gz, July 2010

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Conflict of Interest statement. None declared.

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