Homozygous missense mutation in the *LMAN2L* gene segregates with intellectual disability in a large consanguineous Pakistani family

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ABSTRACT

Background Intellectual disability (ID) is a neurodevelopmental disorder affecting 1%–3% of the population worldwide. It is characterised by high phenotypic and genetic heterogeneity and in most cases the underlying cause of the disorder is unknown. In our study we investigated a large consanguineous family from Baluchistan, Pakistan, comprising seven affected individuals with a severe form of autosomal recessive ID (ARID) and epilepsy, to elucidate a putative genetic cause.

Methods and results Whole exome sequencing (WES) of a trio, including a child with ID and epilepsy and its healthy parents that were part of this large family, revealed a homozygous missense variant p.R53Q in the lectin mannose-binding 2-like (*LMAN2L*) gene. This homozygous variant was co-segregating in the family with the phenotype of severe ID and infantile epilepsy; unaffected family members were heterozygous variant carriers. The variant was predicted to be pathogenic by five different *in silico* programmes and further three-dimensional structure modelling of the protein suggests that variant p.R53Q may impair protein–protein interaction. *LMAN2L* (OMIM: 609552) encodes for the lectin, mannose-binding 2-like protein which is a cargo receptor in the endoplasmic reticulum important for glycoprotein transport. Genome-wide association studies have identified an association of *LMAN2L* to different neuropsychiatric disorders.

Conclusion This is the first report linking *LMAN2L* to a phenotype of severe ARID and seizures, indicating that the deleterious homozygous p.R53Q variant very likely causes the disorder.

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INTRODUCTION

Intellectual disability (ID) is a heterogeneous life-long neurological disorder that affects 1%–3% of the general population.[1 2] Individuals characteristically show delayed developmental milestones, have deficits in the adaptive behaviour and their IQ is less than 70. For this reason, ID represents a serious social and economic burden on families and society.[3] The incidence of ID is higher in male individuals and more than 100 ID-related genes have been identified on the X chromosome, resulting in an estimated contribution of X-linked genes to ID of 10%–15%.[3–5] In addition, recent studies suggest the contribution of more than 1000 autosomal genes to ID aetiology, whereby autosomal recessive ID (ARID) forms are estimated to account for up to 25% of cases.[3–5] Despite a huge advancement of technology in the past years, the identification of ARID-related genes remains challenging.[5]

ID and other autosomal recessive disorders have a high prevalence in inbred populations from the Middle East and South Asia, being one of the major causes of child handicap and mortality.[6–8] The rate of consanguinity in Pakistan is particularly high with approximately 60% of consanguineous marriages, out of which 80% are among first cousins.[6]

We ascertained consanguineous families with severe ID from Baluchistan, Pakistan. In this study we investigated a large consanguineous family with seven severely affected ID individuals in two loops.

METHODS

Patients

A large consanguineous family from the Pashtun population with seven affected individuals (four male and three female) in two loops was ascertained from the district Qilla Abdullah, Baluchistan, Pakistan (figure 1A). At the time of patient recruitment, the age of the affected individuals ranged from 7 to 25 years. Written consent was taken from the participants and from the parents in case of the affected individuals. A consent form was approved by the Institutional Ethical Review Committee at the University of Health Sciences Lahore, Pakistan. We collected 3 mL of peripheral blood in EDTA tubes for DNA isolation. The affected family members were examined by a physician and the family history was assessed. All affected family members alive had a similar phenotype. Five individuals were affected with severe ID and mild epileptic seizures until the age of 5 years. Seizures started in the first year of life and stopped spontaneously without any medication. Typical seizure episodes lasted for 3 to 5 min. Two family members suffered from severe ID and epilepsy and died at the age of 7 and 16 years. They could therefore not be studied (V:10, V:12). One affected male (V:8) also showed aggressive sexual behaviour. The level of ID was estimated by the symptoms observed by the physician: all affected individuals showed a general developmental delay and were completely dependent on others for food, shelter, clothing and cleaning. They could not attend school and even as adults they were able to speak only a few words (figure 1B).

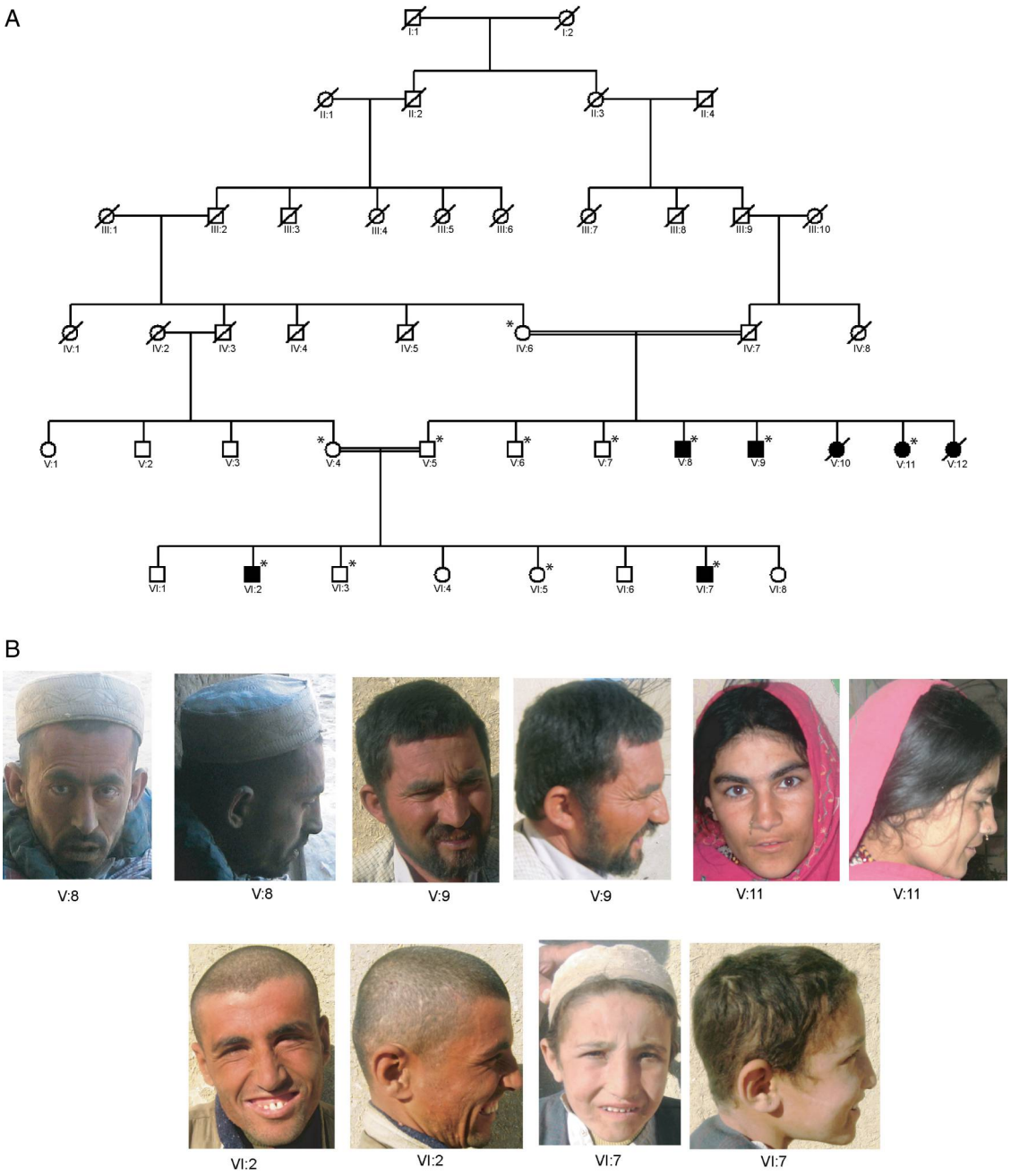

Figure 1 (A) Pedigree of the consanguineous Pakistani family. Squares indicate men and circles indicate women. Individuals with intellectual disability are marked by filled symbols and symbols with cross indicate deceased individuals. *Indicates all the individuals investigated by segregation analysis. (B) Pictures of individuals with intellectual disability.

Whole exome sequencing

Whole exome sequencing (WES) was performed on patient VI:2 and his unaffected parents V:4 and V:5 (figure 1A) on 3 µg of genomic DNA using the Agilent SureSelect Human All Exon V4 (without untranslated regions (UTR)) for exon capture and the Illumina HiSeq 2000 system for sequencing.

The targeted exons in the parent–child trio had an average exon coverage of 126.32× and a median-of-median exon coverage of 115.7×. 99.9% of the targeted exons showed non-zero coverage and 99.5% of the targeted exons had at least 10× coverage.

The raw reads were mapped to the GRCh37 reference sequence[9] using BWA 0.6.2,[10] and duplicates were marked and removed using Picard (http://broadinstitute.github.io/picard/index.html). Quality control details are given in online supplementary table S1. Single nucleotide variants (SNVs) were called using SAMtools and short indels were called using Platypus.[11] Functional classifications of the variants were done using ANNOVAR.[12] Variants were then annotated with SNP database (dbSNP) and 1000 genome data.

Only variants with a read depth of at least 10× and a minimum quality score of 20 were considered for further analysis. Variants with a minor allele frequency >1% in the 1000 genomes project (Phase II) were removed. In addition, a set of 45 exomes from an in-house database were used as a control to remove common variants and sequencing artefacts.

Exonic indels, non-synonymous variants, as well as nonsense and splice site SNVs were filtered further. SNVs were

considered as heterozygous when the variant allele frequency (VAF) was between 15% and 90%. Homozygous variants were defined by a VAF of ≥95% for the minor allele and by a VAF ≤5% for the reference allele. Genotypes predicted by Platypus were used for indels. The genetic mode of inheritance of the variants was determined by combining the parent–child trio data.

In silico prediction programmes (MutationTaster,[13] SIFT,[14] PolyPhen-2,[15] PROVEAN[16] and CONDEL[17]) were used to analyse a putative functional effect. Variants that were predicted as damaging by at least two different programmes were considered for further analysis. Compound heterozygous mutations were only included if both variants were predicted as damaging by at least one prediction tool. H3M2[18] was used to detect runs of homozygosity in the child (VI:2).

Sanger sequencing

Selected variants were validated by Sanger sequencing. PCR amplification was performed with Paq5000 Polymerase (Agilent). PCR products were analysed on an agarose gel, purified and sequenced directly using the DYEnamic ET terminator cycle sequencing kit and the Megabase 1000 DNA analysis system (GE Healthcare).

Isoelectric focusing

Isoelectric focusing of transferrin and α-1-antitrypsin from dry blood spot cards from affected (V:8, V:9, V:11, VI:2, VI:7) and unaffected (IV:6, V:5, V:6, VI:3, VI:6) family members was performed as described.[19]

Homology modelling

Homology modelling of lectin mannose-binding 2-like (LMAN2L) was performed with SWISS MODEL[20] using the crystal structure of vesicular integral-membrane protein 36 (Vip36, also known as lectin mannose-binding 2) from *Canis lupus familiaris* with 62.8% sequence identity with LMAN2L, to build a model (2E6V, Protein Data Bank (PDB)) of LMAN2L wild-type and mutation at amino acid residue 53 (R53 and Q53). These three-dimensional (3D) models were superimposed either to Vip36 (*C. familiaris*) or to the carbohydrate recognition domain of endoplasmic reticulum Golgi intermediate compartment 35 (ERGIC-53-CRD) from *Homo sapiens* (3A4U, PDB), which had 35% sequence identity with LMAN2L in the exoplasmic/luminal domain, to analyse mannose binding and interaction with cargo molecules like multiple coagulation factor deficiency 2 (MCFD2), known to form a complex with ERGIC-53-CRD.

RESULTS

We investigated a large consanguineous family with multiple individuals with a severe form of ID (figure 1A, B) and mild infantile epileptic seizures until the age of 5 years (table 1). All patients were completely dependent on others for food, shelter, clothing and cleaning. They were able to speak only a few specific words. One male patient (V:8) showed aggressive sexual behaviour. We were able to investigate in total five affected and seven unaffected family members (figure 1A). The physical appearance shows no obvious dysmorphic features of the hands, feet and the face.

First, we performed WES of a selected family trio comprising the affected individual (VI:2) and his healthy parents (V:4 and V:5) (figure 1A). After filtering, we obtained 64 variants of which 8 were homozygous, 3 de novo, 47 compound heterozygous and 6 hemizygous (see online supplementary table S2). Homozygosity mapping of the child (see online supplementary figure S1) revealed that all eight homozygous rare variants reside within run of homozygosity (ROH) regions (see online supplementary table S3), six of them (including the *LMAN2L* variant) being located in three large ROHs on chromosome 2 (5.9–14 Mb). Analysis with different *in silico* prediction programmes (MutationTaster, SIFT, PolyPhen2, PROVEAN, CONDEL) revealed 15 putatively pathogenic variants (3 homozygous, 3 de novo, 6 compound heterozygous and 3 hemizygous) in 12 different genes (see online supplementary table S4). For further analysis we considered variants that were (1) predicted deleterious and (2) found in genes expressed in the brain (UniGene, human protein atlas and Allen brain atlas) or (3) residing in highly conserved regions across species. Following these criteria, we selected six variants from five different genes for further validation (table 2).

Three of these genes had already been linked to neurological disorders: *SCN7A* to epilepsy,[21 22] *CSMD1* to autism[23] and schizophrenia[24] and *LMAN2L* to bipolar disorder,[25] schizophrenia[26] and attention-deficit hyperactivity syndrome (ADHS).[27]

To elucidate a putative contribution of the variants to disease, we Sanger-sequenced the five affected individuals (V:8, V:9, V:11, VI:2 and VI:7), their healthy parents (IV:6, V:4 and V:5) and four unaffected siblings (V:6, V:7, VI:3 and VI:5) (figure 1A). Only the homozygous missense variant c.158 G>A; p.R53Q in the *LMAN2L* gene was co-segregating with the phenotype (see online supplementary table S5). *LMAN2L* encodes for a L-type lectin that acts in the glycoprotein trafficking of the endoplasmic reticulum (ER). The variant c.158G>A was found homozygous (A/A) in all five affected individuals and heterozygous (G/A) in the parents and the unaffected siblings (figure 2A, B). This leads to an amino acid substitution (p.R53Q) which is predicted to be pathogenic by five different programmes and is located in a highly conserved protein region (figure 2C). The conservation at position R53 stretches across vertebrates, insects and nematodes. Moreover, the variant was not present in dbSNP (URL: http://www.ncbi.nlm.nih.gov/SNP/) (March 2015 accessed), the 1000 genomes project,[9] the Exome variant server (URL: http://evs.gs.washington.edu/EVS/) (March 2015 accessed) and the browser of the Exome Aggregation Consortium (URL:

Table 1 Summary of clinical information.

Individual ID	Mutation	Gender	Age	Head circumference (cm)	Height (cm)	Epilepsy ≤5 years
V:8	c.158 G>A; p.R53Q	M	25	55.5	NA	+
V:9	c.158 G>A; p.R53Q	M	22	55	168	+
V:11	c.158 G>A; p.R53Q	F	18	54	152	+
VI:2	c.158 G>A; p.R53Q	M	20	55.5	176	+
VI:7	c.158 G>A; p.R53Q	M	7	51.8	109	+

Table 2 Overview of the variants selected for validation by Sanger sequencing. Only *LMAN2L*-R53Q segregated with the phenotype in this family

Gene name	NM_reference	Mode of inheritance	cDNA position	Protein position
ZSWIM6	NM_020928	De novo	c.2289 C>G	p.S763R
SCN7A	NM_002976	Homozygous	c.2867 T>C	p.I956T
PDCD11	NM_014976	Homozygous	c.133 A>G	p.K45E
LMAN2L	NM_001142292	Homozygous	c.158 G>A	p.R53Q
CSMD1	NM_033225	Compound heterozygous	c.6781C>G	p.P2261A
		Compound heterozygous	c.10616A>G	p.Y3539C

http://exac.broadinstitute.org) (March 2015 accessed) which includes more than 8000 exomes from South Asian controls.

The 3D structure of LMAN2L has not yet been experimentally resolved, though the protein shows close homology to other members of the L-lectin family with 35% sequence identity to ERGIC-53 and 63.2% sequence identity to VIP36 (Q12907, Uniprot) in the exoplasmic/luminal domain, also known as the carbohydrate recognition domain. As the 3D structure of Vip36 was only available from *C. familiaris* (P49256, Uniprot; 62.8% sequence identity to human LMAN2L) the 3D structures of *LMAN2L* wild-type (R53) and mutation (Q53) were predicted by homology modelling based on the crystal structure of Vip36 (*C. familiaris*) (figure 3A, B). The carbohydrate recognition domain of VIP36 (2EV6) forms a complex with Ca^{2+} and the sugar mannose,[28] however

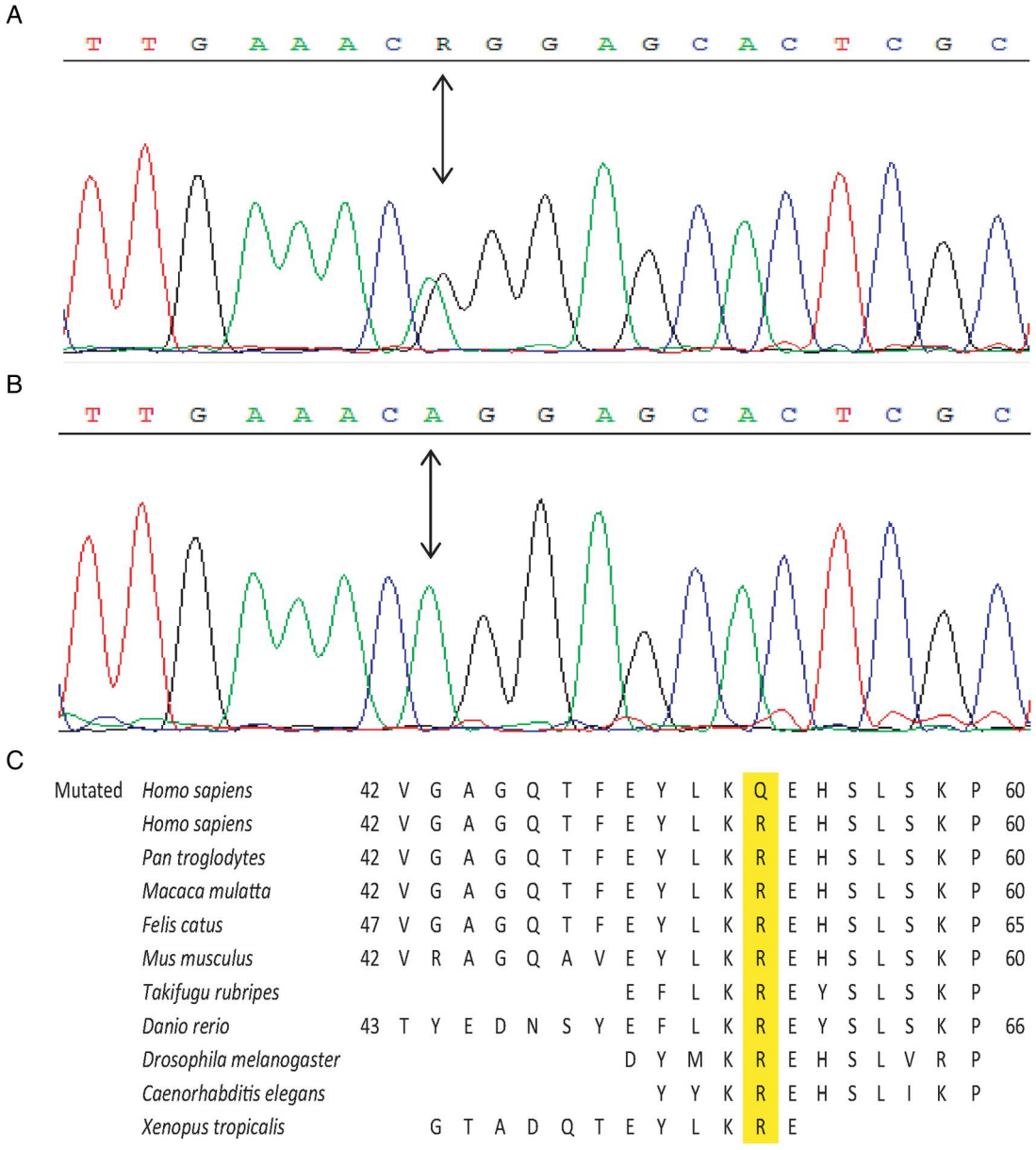

Figure 2 *LMAN2L* sequence of affected and unaffected family members. Chromatograms were obtained by Sanger sequencing. (A) The heterozygous condition for variant c.158G>A was found in parents and other unaffected siblings. (B) The homozygous mutated condition for c.158G>A was identified in all individuals with intellectual disability. (C) Conservation of the *LMAN2L* variant p.R53Q across different species; NP_110432.1; the sequence alignment was taken from the MutationTaster programme.

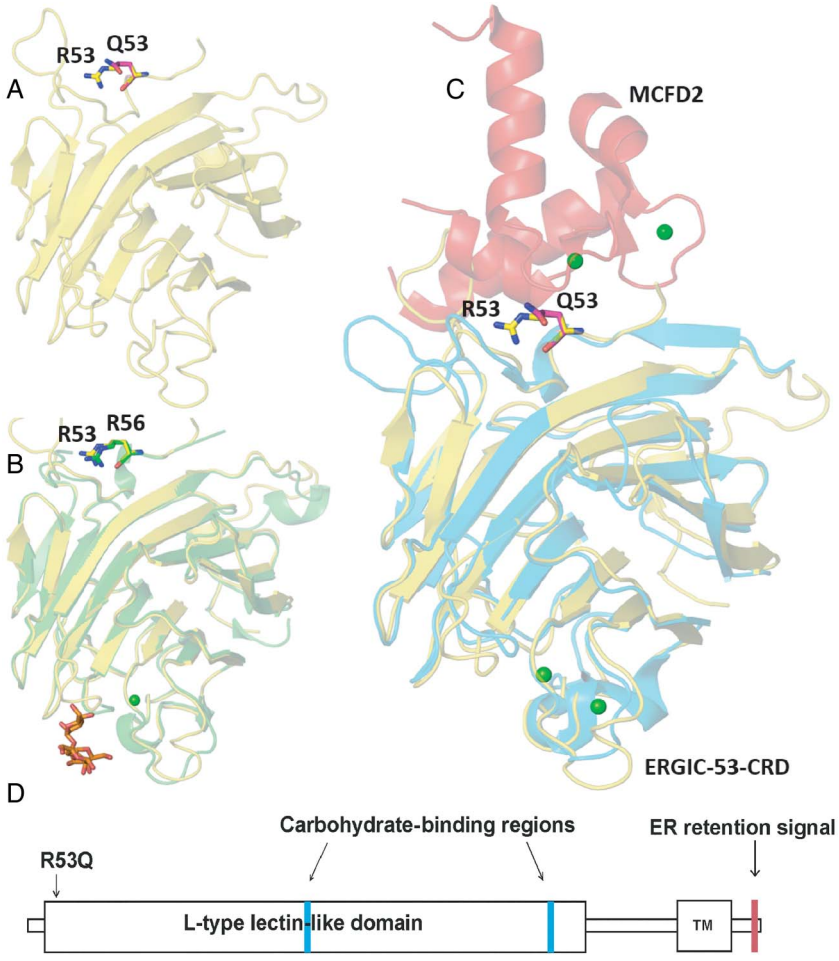

Figure 3 Model of the three-dimensional structure of lectin mannose-binding 2-like (LMAN2L). (A) The protein is shown in cartoon representation with R53 (yellow) in the wild-type protein and Q53 (pink) in the mutant protein in stick representation. (B) The structure of wild-type LMAN2L (yellow) is superimposed on the structure of vesicular integral-membrane protein 36 (Vip36) (Protein Data Bank (PDB) id: 2E6 V, light green) in complex with a Ca^{2+} ion (green sphere) and a mannose molecule (orange sticks). R53 in LMAN2L is conserved in Vip36 as R56 (green sticks). (C) The structure of LMAN2L (yellow) is superimposed onto the structure of the endoplasmic reticulum Golgi intermediate compartment 53-carbohydrate recognition domain (ERGIC-53-CRD) (PDB id: 3A4U, light blue) which is bound to the protein multiple coagulation factor deficiency 2 (MCFD2) (red) to form the cargo receptor. The wild-type (R53, yellow) and mutant (Q53, pink) residues are shown in stick representation. (D) Domain architecture of LMAN2L (Q9H0V9, UNIPROT). The protein consists of a L-type lectin-like and a transmembrane (TM) domain. Carbohydrate-binding regions are shown in blue and the endoplasmic reticulum (ER) retention signal is shown in red.

superimposition of the predicted LMAN2L model revealed that the mannose ligand is located opposed to the N-terminal loop, far from the position of the identified mutation (figure 3B). A comparison of the LMAN2L structure to the ERGIC-53-CRD in complex with soluble calcium-binding protein MCFD2 (3A4U, PDB), identified the p.R53Q mutation to be located exactly at the interface between the two proteins (figure 3C), suggesting a putative impairment in protein–protein interaction.

To find out if the mutated *LMAN2L* has an impact on the glycoprotein biosynthesis, we analysed the glycosylation status of transferrin and α-1-antitrypsin derived from dry blood spot cards by isoelectric focusing (see online supplementary figure S2). Different from serum, the bands of transferrin coming from dry blood normally show a ladder pattern with decreasing intensity from the tetrasialo to the asialo band (see online supplementary figure S2). The comparison of the glycosylation profile between affected (V:8, V:9, V:11, VI:2, VI:7) and unaffected (IV:6, V:5, V:6, VI:3, VI:6) family members did not reveal any obvious differences for both marker proteins (see online supplementary figure S2) and therefore provides no evidence for a general glycosylation deficiency.

DISCUSSION

Using WES we have identified a novel candidate gene for ARID in a large consanguineous family with multiple affected individuals. We identified a homozygous mutation c.158G>A leading to an amino acid substitution p.R53Q in *LMAN2L*, which co-segregates in affected individuals of a large consanguineous Pakistani family with a phenotype of severe ID and seizures. This is the first report linking *LMAN2L* to ARID. *LMAN2L* has been previously associated with different neuropsychiatric disorders including bipolar disorder, schizophrenia and ADHS.[25–27] Together with the results from our study, this suggests that variants in *LMAN2L* may cause various neurodevelopmental and neuropsychiatric disorders including ID and epilepsy.

LMAN2L is located on chromosome 2q11.2 and encodes for a protein of the L-lectin family (comprising ERGIC-53, VIP36 and LMAN2L). It is expressed in various tissues including human brain (eg, cerebral cortex) (URL:http://www.proteinatlas.org/ENSG00000114988-LMAN2L/tissue) (March 2015 accessed). LMAN2L, also named VIPL, is a transmembrane protein located at the ER (figure 3E),[29] which plays a role in the quality control of glycoproteins. It functions as a cargo receptor for glycoproteins and is expected to regulate the exchange of folded and misfolded glycoproteins for transport to the Golgi or to the ubiquitin-proteasome pathway, respectively.[30 31] Different studies have shown that the substitution of a single amino acid can switch the sugar-binding affinity[32] and thereby impair protein function. The homozygous p.R53Q variant identified in our study is located in the N-terminal loop, in the particularly conserved large L-type lectin-like domain in close proximity to the putative homodimerisation or tetramerisation site and therefore probably impairs LMAN2L function. The 3D structure modelling and comparison to homologous proteins suggests that the mutation may impair protein–protein

interaction. Even though very little is known about the interaction partners of LMAN2L, we speculate that the mutation may impair multimer formation, the interaction with a yet unknown protein or may influence the recently hypothesised interaction with ERGIC-53.[29] A functional impairment of LMAN2L may result in an impaired secretion and degradation of its cargo proteins.

Mutations in another glycoprotein quality-control protein, endoplasmic reticulum mannosyl-oligosacchride 1,2-alpha-mannosidase (MAN1B1), have been reported in Pakistani families with ARID and epilepsy; however, the phenotype was generally milder and only some patients presented epilepsy.[33] The protein MAN1B1 introduces degradation signals to misfolded glycoproteins in the ER which is then recognised by lectins leading to proteasomal degradation. Although MAN1B1 and LMAN2L play a role in the quality control of glycoproteins, they are proteins with different functions: MAN1B1 acts as mannosidase,[34] whereas LMAN2L functions as cargo receptor.[31] *MAN1B1* mutations result in impaired glycosylation patterns of transferrin[35] whereas this is not the case for LMAN2L. Even though LMAN2L plays a role in the transport of glycoproteins, no abnormalities of α-1-antitrypsin and transferrin glycosylation were detected, similar to other genes directly linked to the glycosylation machinery (eg, GCS1-CDG,[36] PGM3-CDG,[37] NgBR-CDG,[38] GMPPA-CDG[39] and N33/ TUSC3-CDG[40]) which also do not lead to hypoglycosylation of transferrin. Patients suffering from N33/ TUSC3-CDG, for example, also present developmental delay and non-syndromic ID as the main clinical features.

As the therapeutic options for patients with ARID and epilepsy are currently limited, determining new pathways or targets for therapy is important. The discovery of this novel gene *LMAN2L* and other proteins of the glycoprotein quality-control pathways may be helpful in developing therapeutics for ID and epilepsy.

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Contributors RR performed the experimental work, data analysis and wrote the manuscript; MA initiated the development of this project; NP, SW, MS performed and analysed the NGS data; CT carried out the glycosylation analysis; GM performed the homology modelling of LMAN2L protein; RCW contributed to the 3D structure analysis and data interpretation; GAR supervised and supported this project and SB contributed to the interpretation of results and wrote the manuscript.

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Competing interests None declared.

Patient consent Obtained.

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