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ARHGEF9 disruption in a female patient is associated with X linked mental retardation and sensory hyperarousal

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ABSTRACT

Introduction: We identified a female patient with mental retardation and sensory hyperarousal. She has a de novo paracentric inversion of one X chromosome with completely skewed inactivation of the normal X chromosome.

Objective: We aimed to identify whether a single gene or gene region caused her cognitive and behavioural impairment and that of others.

Results: Fluorescent in situ hybridisation (FISH) showed that the centromeric breakpoint disrupts a single gene: ARHGEF9 (CDC42 guanine nucleotide exchange factor (GEF) 9). The telomeric break lies in a gene poor region. We also found that the levels of the ARHGEF9 transcript from the patient are 10-fold less than those found in control samples. Consequently, we sequenced the coding exons and intron/exon borders of the ARHGEF9 gene in 99 probands from families with X linked mental retardation (XLMR) and 477 mentally retarded males in whom a diagnosis of Fragile X syndrome had been excluded. We did not identify any pathogenic changes; however, we did identify intronic nucleotide changes that might alter splicing.

Conclusion: ARHGEF9 encodes a RhoGEF family protein: collybistin (hPEM), which is highly expressed in the developing and adult brain. Collybistin can regulate actin cytoskeletal dynamics and may also modulate GABAergic and glycinergic neurotransmission through binding of a scaffolding protein, gephyrin, at the synapse. This potential dual role may explain both the mental retardation and hyperarousal observed in our patient. While ARHGEF9 appears to be an uncommon cause of mental retardation in males, it should be considered in patients with mental retardation and sensory hyperarousal.

Mental retardation is a significant health issue for the affected individuals, their families, and society. An estimated 10% of mental retardation is believed to be attributed to genes on the X chromosome.[1 2] While studies vary in their measurement tools and intelligence quotient (IQ) classification, mental retardation is estimated to affect up to 3% of the population with societal cost estimates exceeding those for stroke, heart disease and cancer combined.[3 4] Further epidemiological data have shown that males suffer from all degrees of mental retardation at least 20% more often than females.[5] This variance is conjectured to arise, in part, from mutations in the genes on the X chromosome. While researchers speculate that the X chromosome may contain a higher proportion of genes encoding proteins involved in cognition than other chromosomes, it is certainly true that the singularity of the X chromosome in males simplifies the search for mutations. Furthermore, the utilisation of disease associated chromosomal rearrangements involving the X chromosome has been a fruitful strategy for identifying novel X linked mental retardation (XLMR) genes, including oligophrenin-1, TM4SF2, ARHGEF6, and ZNF741.[6–9]

The number of mental retardation genes identified on the X chromosome has significantly increased in recent years, with 52 syndromic and 25 non-syndromic genes identified to date.[10] The designation of syndromic mental retardation (S-XLMR) is based on whether abnormalities in addition to mental retardation are found on physical examination, laboratory investigations, or radiological studies. If no additional abnormalities are found, the classification of non-syndromic X linked mental retardation or NS-XLMR is used. While numerous genes for XLMR have been discovered, estimates suggest that over 100 genes on the X chromosome may contribute to XLMR alone, which points to an ongoing need for investigation.[2]

Herein we report our findings implicating ARHGEF9 as a novel candidate gene for XLMR with the behavioural phenotype of sensory hyperarousal. ARHGEF9 was identified through mapping the breakpoints of a de novo paracentric X chromosome inversion (46, X, inv (X) (q11.1q27.3) in a cognitively impaired female patient with hyperarousal to noise. Interestingly, ARHGEF9 was previously implicated in XLMR in a patient with associated hyperekplexia and epileptic encephalopathy.[11] Thus, ARHGEF9 should be considered in patients with NS-XLMR with hyperarousal, hyperekplexia, and/or epilepsy.

METHODS

Case report

The proband is the only child of healthy, non-consanguineous parents. The pregnancy was uncomplicated and her family history is non-contributory. Growth parameters at birth and at present are within normal range and in alignment with her family constellation. Her parents first became concerned about her development at 18 months due to symptoms of hyperarousal to noise and social situations. These symptoms have persisted and severely limit her routine activities and family life. Following this initial concern, global developmental delay was suspected. She

walked at 13 months, began to bicycle at 8 years, and continues to have fine and gross motor incoordination. Her first single words began at 2 years, and at 9 years she remained unable to read or to perform simple addition. As a teenager, she continues to be unable to tolerate a multitude of auditory stimuli which engender autonomic hyperarousal with "pounding heartbeat and sweating". These stimuli are then actively avoided. For example, her parents are unable to take her to any event featuring fireworks and she will cross the street and cover her ears when any type of cannon (real or model) is encountered. She has always been considered shy due to limited eye contact and social initiative/interaction. She has hyperactivity and impulsivity in multiple contexts with withdrawal and hyperactivity scores above diagnostic threshold on the Behavior Assessment System for Children, 2nd edition (BASC-2) and Conner's parent rating scale-revised, respectively. Neuropsychological testing with the Wechsler Intelligence Scale for Children, 4th edition (WISC-IV) at 15 years of age reveals performance in the mild to moderate mental retardation range: verbal comprehension index 55, perceptual reasoning index 51, working memory index 65, processing speed index 62, and a full scale IQ of 48.

Her general physical examination was unremarkable. She has slightly dysarthric speech and difficulty with smooth eye pursuit. Her motor examination was characterised by mild bilateral lower extremity spasticity with brisk reflexes and extensor plantar responses. She had mild incoordination on pointing and a wide-based gait. Metabolic laboratory results and FMR1 genetic testing were within normal limits and the magnetic resonance imaging of her brain was not diagnostic. Karyotype analysis showed a de novo paracentric inversion (46, X, inv (X) (q11.1q27.3)) on one X chromosome. Consent was obtained from the proband and her parents for participation in this study in accordance with the University of California committee on human research protocol.

X chromosome inactivation analysis

Replication studies were performed on freshly isolated phytohaemagglutinin (PHA) stimulated T lymphocytes from the proband after 5-bromodeoxyuridine (BrdU) incorporation.[12]

Fluorescent in situ hybridisation (FISH)

DNA (1 μg) isolated from the bacterial artificial chromosome (BAC) clones was labelled by nick-translation with Cy3-dUTP (Amersham Life Sciences PA53022, Little Chalfont, Bucks, UK) in 50 μl reactions (table 1). Labelled probes were then hybridised to metaphase spreads from the patient's lymphocyte Epstein Barr virus (EBV) transformed cell line and a normal control. After hybridisation, the chromosomes were stained with a DAPI-Antifade solution. More than 10 metaphase and interphase cells were assessed for each probe by visualisation using a Zeiss Axioplan microscope with a Photometrics Synsys camera using the Applied Imaging MacProbe 4.3 image capture system.

In silico analysis

The human reference sequence, Build 36.1, was used as a genome resource. The localisation data from the FISH studies were mapped to determine candidate genes for further analysis using the UCSC genome browser (http://genome.ucsc.edu). All expressed sequence tags (ESTs) were investigated for open reading frames using the Genamics Expression program (http://genamics.com/expression/index.htm).

RNA preparation and quantitative RT-PCR

Fresh blood was collected from the patient directly into a PAXgene RNA blood tube.[13] Total RNA was then isolated according to the manufacturer's instructions. Total RNA from pooled human peripheral blood leucocytes (250 male/female caucasian population) was also obtained for comparison (Clontech, cat# 636580, Mountain View, California, USA). ARHGEF9 and GAPDH PCR oligonucleotide primers and probes were obtained from Applied Biosystems (Foster City, California, USA) (table 2). Reverse-transcriptase (RT) polymerase chain reaction (PCR) was conducted in triplicate with 20 μl reaction volumes of 1X Taqman buffer (1X Applied Biosystems PCR buffer, 20% glycerol, 2.5% gelatin, 60 nM Rox as a passive reference), 5.5 mM $MgCl_2$, 0.5 mM each primer, 0.2 μM each deoxynucleotide triphosphate (dNTP), 200 nM probe, and 0.025 unit/μl AmpliTaq Gold (Applied Biosystems) with 5 ng cDNA. A large master mix of the above mentioned components (minus the primers, probe, and cDNA) was made for each experiment and aliquoted into individual tubes, one for each cDNA sample. The cDNA product was subsequently added to the aliquoted master mix and transferred to a 384 well plate where upon the primers and probes were included. PCR was conducted on the ABI 7900HT (Applied Biosystems) using the following cycle parameters: one cycle of 95° for 10 min and 40 cycles of 95° for 15 s, 60° for 1 min.

Analysis, to determine the cycle threshold (Ct) values of each reaction, was carried out using the SDS software supplied with the ABI 7900HT (version 2.3). Ct values were determined in triplicate for patient and control samples then averaged. PCR efficiencies were measured for all custom assays and were ⩾90%. For the purpose of comparing ARHGEF9 expression between the patient and control samples, we normalised the ARHGEF9 Ct to the GAPDH Ct for each sample source. ΔCt was computed by simple subtraction [ΔCt = Ct (test locus)–Ct (control locus)] and relative fold difference was calculated for each primer/probe combination as $2^{-\Delta Ct} \times 100$.

Fine tiled X chromosome arrays

DNA was isolated from the patient and her parents by standard protocol (DNeasy, Qiagen, Valencia, California, USA). The NimbleGen Systems ultra high resolution comparative genomic hybridisation (CGH) microarray platform was used for the detection of copy number polymorphisms. Specifically, the human CHR X Fine-Tiling array, which contains 385 000 oligonucleotide probes on a single glass slide, was used. The median probe spacing is 106 base pairs with isothermal probes, T_m 76°C and probe length ranging between 45mer and 85mer. The patient's DNA sample was separately compared to the DNA of her mother and father.

Mutation screening of ARHGEF9 in probands with mental retardation by direct sequencing

DNA was obtained from 576 males with mental retardation from the Greenwood Genetics Center in Greenwood, South Carolina, USA. All individuals had agreed to the use of their DNA for research purposes through the Greenwood Genetics Center. These DNA samples represent three populations of males with NS-XLMR. The first group consisted of probands from XLMR families with linkage to the region of ARHGEF9 in Xq11.1 (n = 16); the second cohort consisted of males with a family history of XLMR but no linkage data (n = 83); and the third was made up of males without a specific family history of mental retardation (n = 477). All patients were negative for

Table 1 Bacterial artificial chromosome (BAC) position on the X chromosome with fluorescent in situ hybridisation (FISH) results

BAC clone	Position (bp)	Hybridisation signal
Centromeric BAC		
RP11-246O11	61712881–61869854	Proximal
RP11-59G10	62384931–62541177	Proximal
RP11-326H7	62666941–62818681	Proximal
CTD-2129K15	62684870–62857731	Proximal
RP11-570J18	62819644–62876218	Spanning
RP11-73M8	62846957–63002616	Spanning
RP11-151A2	62876160–63041215	Distal
RP11-213M6	62925015–63088068	Distal
RP11-153P2	64783602–64949326	Distal
Telomeric BAC		
RP11-414E19	140091955–140285194	Proximal
RP11-469P18	140421869–140603155	Proximal
RP11-125M11	141511402–141675161	Proximal
CTD-2045C2	141906264–142009640	Spanning
CTD-3095F11	141954729–142066292	Spanning
RP11-179F2	141954733–142114193	Spanning
RP11-346M2	142339338–142513687	Distal
RP11-75E18	142754841–142919725	Distal
RP11-100N24	147886638–148058378	Distal

expansion of the Fragile X trinucleotide repeat. Ten exons, including the intron/exon borders, were amplified and sequenced from all samples based on the representative mRNA transcript: AB007884. Primers were designed to include donor, acceptor, and branch point regions (table 3). Sequencing was performed on the ABI 3730I using standard dye terminator protocols. All variations from the posted sequence were re-sequenced and checked against the dbSNP database (build 124). Potentially pathogenic alterations were then investigated in male controls (n = 282) using restriction enzyme analysis. The Automated Splice Site Analyses web interface tool and the Maximum Entropy Splice Site calculator were utilised to predict which sequence changes might alter splicing of the heterogeneous nuclear RNA.[14 15] To predict the effect of the alterations on the ARHGEF9 protein both the PolyPhen (Polymorphism Phenotyping) program (http://genetics.bwh.harvard.edu/pph/) and the SIFT (Sorting Intolerant From Tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html) were also utilised.[16 17]

RESULTS

Mapping of the translocation breakpoints using FISH

The initial analysis of the karyotype showed a paracentric X chromosome inversion at Xq13.1 and Xq26.3. BAC clones were selected to flank both regions (table 1). The intervals were narrowed until at least one BAC clone that spanned each breakpoint was identified. At the breakpoint near the centromere, two BAC clones, RP11-570J18 and RP11-73M8, were identified that hybridised to centromeric and telomeric regions on the derivative X chromosome. We confirmed and narrowed this interval to approximately an 18 487 base pair region using two additional BACs, CTD-2129K15 and RP11-151A2. This region is between the first and third exons of the ARHGEF9 gene on Xq11.1 (fig 1).

A similar strategy was employed for the telomeric breakpoint. BAC clones CTD-3095F11, CTD-2045C2, and RP11-179F2 span the telomeric breakpoint and share an overlapping region of 54 911 bp which maps to Xq27.3 (fig 1). There are no known genes in the defined region nor is there evidence of conserved elements. The two ESTs in the region, CD558550 and AI283614, do not have significant open reading frames as determined by the Genamics Expression software (http://genamics.com/expression/index.htm). As shown in fig 1, the telomeric breakpoint lies within 50 kb downstream of SPANX-N4, a protein expressed exclusively in normal testis and certain tumours.[18]

Fine tiled X chromosome arrays

The DNA from the patient was hybridised to her maternal and paternal DNA independently to detect copy number gain or loss of genomic material on the X chromosome. There were no de novo copy number changes or evidence for gain or loss of genetic material detected near either breakpoint (data not shown). This array technique has a level of resolution of approximately 4 kb and would not be able to definitively detect losses or gains of genetic material below this level of resolution.

X chromosome inactivation analysis

The X inactivation pattern for the proband based on late replication studies was determined from freshly isolated PHA stimulated T cell lymphocytes. BrdU incorporation in 100 studied metaphases of T lymphocytes showed that the normal X chromosome was late replicating in all 100 cells examined (data not shown). This finding of completely skewed X

Table 2 Primers and probes used to analyse ARHGEF9 and GAPDH RNA transcripts*

Regions	Primers	Sequence
ARHGEF9 (exon 2–3)	Forward (5′–3′)	n/a
	Reverse (5′–3′)	n/a
Hs00209514_m1	Probe	n/a
	Amplicon size (bp)	68
ARHGEF9 (exon 8–9)	Forward (5′–3′)	n/a
	Reverse (5′–3′)	n/a
Hs01003485_m1	Probe	n/a
	Amplicon size (bp)	83
GAPDH	Forward (5′–3′)	ATTCCACCCATGGCAAATTC
	Reverse (5′–3′)	TGGGATTTCCATTGATGACAAG
	Probe	Fam-TGGCACCGTCAAGGCTGAGAACG-bhq
	Amplicon size (bp)	72

*PCR primer and TaqMan probe sequences purchased from Applied Biosystems. n/a = not available as this is proprietary information.

Table 3 Primers for direct sequencing of ARHGEF9 from DNA templates

Exon	Amplicon size (bp)	Forward (5′–3′)	Reverse (5′–3′)	Anneal temp (°C)
1	306	TAGTCCTCCTGCTTGTCAATG	AATAGGCAACTATACGCGCAG	60
2	347	AAGTAGTAGAGCTCTGTGGGTGG	AAACAGGAGAACAGAGGCTGG	60
3	362	TTTTCTACTTGCCTGGAAACC	CTATGTGCCCTTTCCAACATC	60
4	350	TTCTTGGGACCAAAATGAGG	AGACAGCTCAAACGCTCCTTC	60
5	636	TTACCCTTCTTTTACAGAATGTCC	GTTGGCTATGAGGCAATCAAG	55
6	296	GTGGATGTTAAGATTGGCACC	TTTCTCTTCACCTGTCCTTAGC	60
7	301	CCTATCCCAGTCTATGCCCAG	GGGATGATGAAGAGGAGGGTC	60
8	410	GGTGACAGTGTTAGCATTCTGC	AAAATGGAAACCAATTCAAACC	60
9	446	TCCATTAACAGACTCTTAGCCC	CCCAAGTAAACCTCAGCACAG	60
10	349	GAGTGGTCACTGGGTAATTTGAG	AATTTCAACAGTGCTTCTCCG	60

inactivation in favour of the abnormal X chromosome correlates with the observed XLMR phenotype in this female patient.

Assessment of ARHGEF9 transcript levels using quantitative PCR

Based on both the FISH and replication studies implicating ARHGEF9 as the explanation of the patient's mental retardation and sensory hyperarousal, we sought to compare the steady state level of transcription from this gene in the blood from patient and controls using quantitative RT-PCR (table 4). Both the patient and control mRNA have high and relatively similar levels of transcript for the control gene, GAPDH. By contrast, the patient's mRNA sample contains 9% of the ARHGEF9 transcript relative to the pooled control sample.

ARHGEF9 mutation screening

Sequencing of the ARHGEF9 gene in 576 males with non-fragile X-linked mental retardation identified two alterations within a predicted branch point sequence, two non-synonymous alterations, and two synonymous alterations (table 5). Based on information theory binding site analysis, the branch point change, IVS7-56 C>G, is predicted to reduce the branch point binding affinity to 10%. The IVS7-54 C>T may also affect the same branch point but only reduces affinity to 66% of its original strength. By contrast, the synonymous alterations (c.558G>A and c.1092C>T) do not appear to affect splice site binding.[14] The identified non-synonymous variant at amino acid position 10 that changes an isoleucine to valine (p.I10V) was

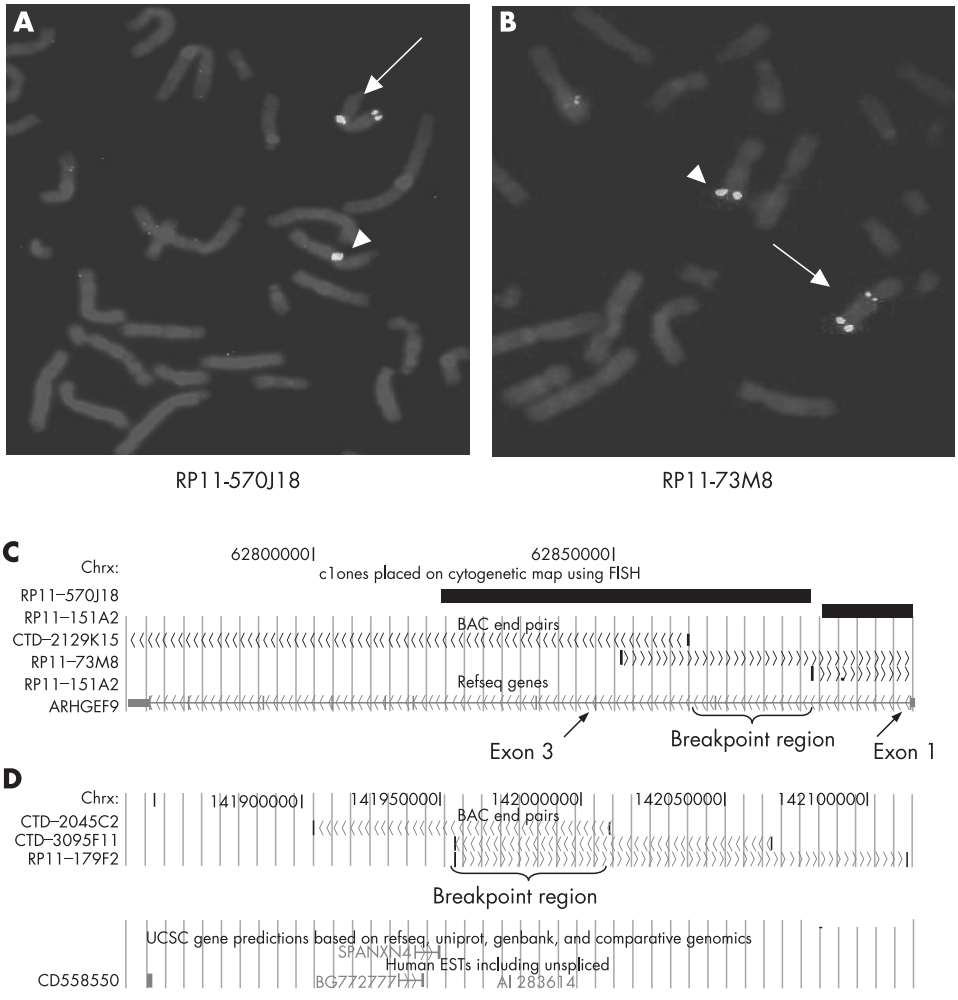

Figure 1 Fluorescent in situ hybridisation (FISH) mapping of the centromeric and telomeric breakpoints with bacterial artificial chromosomes (BACs). (A) FISH with BAC RP11-570J18 shows equal hybridisation at two loci on the inverted X chromosome (arrow) relative to one locus on the unaffected X (arrow head). (B) FISH with RP11-73M8 reveals unequal hybridisation at two loci confirming and narrowing the breakpoint interval (arrow); unaffected X (arrow head). (C) Bracketed region on genome schematic (Xq11.1) outlines estimated breakpoint region as defined by these FISH results (BACs listed in table 1). The centromeric breakpoint lies between ARHGEF9 exons 1–3. (D) Bracketed region on genome schematic (Xq27.2-27.3) depicts estimated telomeric breakpoint region. (FISH data not shown).

Table 4 Relative ARHGEF9 mRNA expression normalised to GAPDH

	ARHGEF9 (exon 2–3)		ARHGEF9 (exon 8–9)	
mRNA source	Observed Ct (SD)	Ratio*	Observed Ct (SD)	Ratio*
Patient	32.41 (0.33)	0.13	34.75 (0.07)	0.02
Control	26.94 (0.04)	1.49	29.70 (0.08)	0.22

*Normalised to GAPDH Ct values.

found to be present in 0.4% (1/282) of the normal male X chromosomes. The non-synonymous substitution of a valine for an isoleucine at position 402 (p.I402V) was not present in the 282 normal male X chromosomes. PolyPhen analysis predicted that the p.I402V variant is benign with the same amino acid change occurring in Mus musculus (accession number NM_001033329.2). In addition, SIFT analysis predicted that the isoleucine to valine change at position 402 can be tolerated (data not shown). Based on these findings, the synonymous and non-synonymous alterations are unlikely to cause XLMR; however, the branch point changes may alter splicing and affect the resulting mRNA and protein products.

DISCUSSION

ARHGEF9 is associated with NS-XLMR in a female with a paracentric X chromosome inversion. By fine mapping the breakpoints of this inversion, we discovered that the centromeric breakpoint is contained within the ARHGEF9 gene while the telomeric breakpoint is in a gene poor region. Quantitative RT-PCR using two different ARHGEF9 primer pairs demonstrated that the proband's expression of ARHGEF9 was 10-fold less than the expression in a pooled sample of mRNA from 250 phenotypically normal males and females. This finding suggests that the centromeric breakpoint which transects the ARHGEF9 gene in our patient has led to decreased transcription of the mRNA from this gene. The observation that she appears to have completely skewed X inactivation with expression from her inverted X chromosome helps explain why this female patient has phenotypic expression of mental retardation rather than remaining an asymptomatic carrier. Complete skewing of X chromosome inactivation has been documented in a number of mentally retarded females.[19 20] However, we now also recognise that some genes on the X chromosome can be partially expressed from an otherwise inactive X chromosome.[21] The residual expression that we have documented with quantitative PCR in her peripheral leucocytes may result from varied X inactivation pattern across cell populations (X inactivation was measured in PHA activated T cells, while the quantitative measure of ARHGEF9 mRNA was from a heterogeneous mixture of blood cells) or from partial activity of the ARHGEF9 gene from her normal X chromosome. Collectively, these data strongly support a causative role for ARHGEF9 in the proband's mental retardation and lead us to sequence the ARHGEF9 exons and intron/exon borders in 99 probands from families with XLMR and 477 males with non-fragile X-linked mental retardation. Although we did not find clear inactivating mutations in the coding sequence, we found two nucleotide changes which may alter splicing of the 7th intron by affecting the branch site affinity.

ARHGEF9, which codes for a protein alternately known as PEM-2, HPEM-2, and collybistin, is a strong candidate for modulating cognitive function based on structural and functional work of this and similar protein products.[22] It is highly expressed in neurons in multiple regions within the brain during development and adulthood.[11] Structurally, collybistin contains an N-terminal SRC homology 3 (SH3) domain followed by tandem DBL homology (DH), pleckstrin homology (PH) domains, and a C-terminal proline-rich sequence. This protein structure identifies ARHGEF9 as a member of the Rho GTPase activator family (RhoGEF). RhoGEFs are responsible for the activation of Rho-family GTPases and are responsive to a multitude of extracellular stimuli. These proteins are known to affect the plasticity and polarity of the eukaryotic actin cytoskeleton and to alter cell signalling transduction pathways.[23–25]

Table 5 ARHGEF9 polymorphisms found in males with mental retardation

Region	Base change	Predicted mRNA or protein alteration	Patients (n = 576)	Male controls (n = 282)
Missense				
Exon 2	c.30A>G	p.I10V	3	1
Exon 8	c.1204A>G	p.I402V	1	0
Silent				
Exon 4	c.558G>A	p.E186	1	n/t
Exon 8	c.1092C>T	p.G364	1	n/t
Branch point changes				
IVS7-56 C>G	c.1056-56C>G	r.spl?	3	n/t
IVS7-54 C>T	c.1056-54C>G	r.spl?	2	n/t

r. spl? = potential receptor splice site alteration; n/t = not tested.

Collybistin is also believed to play an important role in inhibitory neurotransmission via its role in the synaptic localisation of the glycine and GABA(A) receptors. Collybistin appears to translocate glycine receptors and specific subtypes of GABA(A) receptors to the cell membrane through its interaction with the multidomain protein gephyrin.[11 26–28] An absence or disruption of collybistin thus would be expected to result in impaired synaptic inhibition. A previously described mutation did lead to the clinical findings of hyperekplexia and seizures in a male carrying a missense mutation (p.G55A) in the ARHGEF9 gene.[11] The p.G55A missense mutation appears to confer a more severe phenotype than the null mutation observed in our patient. Our patient may be partially rescued by the intact ARHGEF9 on her normal X chromosome. In addition, as suggested by Harvey *et al*, collybistin with the p.G55A mutation appears to form large somatic and dendritic aggregates with gephyrin. While a null mutation would negatively affect translocation, it would not sequester the available gephyrin.[11] The sensory hyperarousal observed in our higher functioning female patient may be caused by a similar yet less severe alteration of the glycine and GABA(A) receptor localisation, suggesting a rational direction for chemically targeting the inhibitory neurotransmitter system.

Three other genes which have been implicated in X-linked mental retardation have functions similar to ARHGEF9: OPHN1, PAK3, and ARHGEF6. This underscores the importance of regulation of small G proteins in cognition and synaptic plasticity.[6 7 29] Oligophrenin-1 is a RhoGAP protein whose mutation might lead to constitutive activation of GTPase targets, thus affecting cell migration and outgrowth of axons and dendrites. PAK proteins appear to function as links between Rho GTPases and actin cytoskeleton. ARHGEF6, like ARHGEF9, is specifically a member of the RhoGEF family. Similar to our report, ARHGEF6 was implicated in mental retardation through the study of a male with a chromosomal rearrangement. The authors then found an ARHGEF6 acceptor

Key points

- ARHGEF9 is associated with NS-XLMR by truncation from a paracentric inversion and a 10-fold reduction in peripheral blood mRNA levels in a female with moderate MR and sensory hyperarousal.
- Sequencing of the ARHGEF9 gene in 576 males with mental retardation (83 were members of families with X-linked MR, and all were excluded for Fragile X) identified two predicted branch point sequence alterations and two non-synonymous alterations, but no clear inactivating coding mutations.
- ARHGEF9 should be considered in males with severe mental retardation, seizures and hyperekplexia, and in females with mild mental retardation and pronounced sensory hyperarousal.

splice site mutation which segregated with disease in a large Dutch family (MRX46). While our sequencing effort suggests that ARHGEF9 is an uncommon cause of mild to moderate XLMR in males, it and similar genes certainly play an important role in cognition and behaviour. ARHGEF9 should be considered in males with severe mental retardation, seizures and hyperekplexia, and in females with mild mental retardation and pronounced sensory hyperarousal.

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Competing interests: None.

Informed consent was obtained for publication from the parents of the individual who is described in this report.

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