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Loss of *ZDHC15* expression in a woman with a balanced translocation t(X;15)(q13.3;cen) and severe mental retardation

Mahmoud Reza Mansouri¹, Lena Marklund¹, Peter Gustavsson², Edward Davey¹, Birgit Carlsson¹, Catharina Larsson², Irene White³, Karl-Henrik Gustavson¹ and Niklas Dahl^{*,1}

¹Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden; ²Department of Molecular Medicine, Karolinska Hospital CMM L8, Stockholm, Sweden; ³Department of Clinical Genetics, Karolinska Hospital, Stockholm, Sweden

X-linked mental retardation (XLMR) affects one in 600 males and is highly heterogeneous. We describe here a 29-year-old woman with severe nonsyndromic mental retardation and a balanced reciprocal translocation between chromosomes X and 15 [46,XX,t(X;15)(q13.3;cen)]. Methylation studies showed a 100% skewed X-inactivation in patient-derived lymphocytes indicating that the normal chromosome X is retained inactive. Physical mapping of the breakpoints localised the Xq13.3 breakpoint to within 3.9 kb of the first exon of the *ZDHC15* gene encoding a zinc-finger and a DHHC domain containing product. Expression analysis revealed that different transcript variants of the gene are expressed in brain. *ZDHC15*-specific RT-PCR analysis on lymphocytes from the patient revealed an absence of *ZDHC15* transcript variants, detected in control samples. We suggest that the absence of the *ZDHC15* transcripts in this patient contributes to her phenotype, and that the gene is a strong candidate for nonsyndromic XLMR.

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Introduction

X-linked mental retardation (XLMR) affects approximately one in every 600 males and can be categorised as syndromic with a specific clinical or metabolic phenotype associated with the disease, or nonsyndromic in which the only consistent clinical feature is mental retardation (MR).^{1,2} The latter category accounts for approximately two-thirds of all XLMR patients.³ To date, 20 genes have successfully been identified in nonsyndromic XLMR.⁴

However, mutations in these genes are rare and it is estimated that close to 100 genes might be involved in nonsyndromic XLMR.^{5,6}

Molecular characterisation of *de novo* chromosomal abnormalities has proven to be useful in the search for candidate XLMR genes. Genes on the X chromosome disrupted by balanced X;autosome translocations in female subjects with MR are good candidate genes as they usually have only one functional copy.^{7,8} The normal allele is inactive in most female carriers of such balanced translocations due to skewed X-inactivation with the derivative X chromosome retained active.⁹

In this study, we investigated the translocation breakpoints in a female patient with severe, nonsyndromic MR who carries a *de novo* balanced translocation t(X;15). Based on X-inactivation and gene expression studies, we show

*Correspondence: Professor N Dahl, Section of Clinical Genetics, Department of Genetics and Pathology, The Rudbeck laboratory, Uppsala University Children's Hospital, S-751 85 Uppsala, Sweden.

Tel: +46 18 6112799; Fax: +46 18 554025;

E-mail: niklas.dahl@genpat.uu.se

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that the breakpoint disrupts the transcription of a gene containing a zinc-finger DHHC domain (*ZDHHC15*) [AK056374] in this patient. We propose that our finding might be of functional importance for a subset of individuals with nonsyndromic XLMR.

Materials and methods

Patient

The patient was a 29-year-old female with the karyotype 46,XX,t(X;15)(q13.3;cen) originally reported by Gustavson *et al* in 1984.¹⁰ She was delivered after 39 weeks of gestation by caesarean section with a birth weight of 3100 g, height of 49 cm, head circumference of 34 cm and an Apgar score 9. Her parents are unrelated and healthy and there is no family history of congenital birth defects or MR. Both parents and her brother have normal karyotypes. In the neonatal period, the patient displayed muscular hypotonia, which persisted until 12 years of age. Her height remained in the normal range during childhood but her weight increased to +3SD at 2 years of age. Mild obesity was observed at 3 years of age which disappeared in adulthood. Epileptic seizures were observed from 4 years of age. At 17 years of age, her height was 164 cm (± 0 SD), her weight 55.7 kg (± 0 SD) and she had a head circumference of 55 cm (± 0 SD). At age 19 years, her clinical picture was characterised by severe psychomotor retardation with an IQ of 30. She has a dysmorphic facial appearance with a small nose, epicanthal folds, a high arched palate, large front teeth, low posterior hair line, small hands with clinodactyly of fifth fingers and small feet in planovalgus position. She is unable to speak and she expresses herself with sounds and gestures and needs help with her daily care including bathroom visits, feeding and dressing. She has always been good-natured but reserved. She has difficulties with reciprocal social interactions and she is emotionally unstable with rage-type responses. This study was approved by the appropriate Swedish review board and an informed consent was obtained from the parents.

Fluorescence *in situ* hybridisation (FISH) analysis

EBV-transformed B lymphocytes from the patient were used to prepare metaphase chromosomes on microscope slides for FISH and mini-FISH¹¹ analysis as described.¹² The Xq13.3-specific BAC clones RP11-145B3, RP11-17P24, RP11-311P8, RP11-262B13, RP11-451L9, RP11-236O12 and RP11-324B6 (Roswell Park Cancer Institute) were obtained from RZPD-Germany. The chromosome 15 centromere-specific probe D15Z1 was a gift from professor Bradley N White, Queens University, Canada and an α -satellite probe for all centromeres was obtained from Qbiogene. The mini-FISH probe ZDHHC15mf was amplified using the Advantage 2 PCR kit (Clontech) with the primers 5'-CCTTTCCAAAACCCAGGTTGC-3' and 5'-GACGGATCACCCCTCCGCTCC-3'.

Southern blot analysis

DNA from the patient and healthy controls was prepared from peripheral blood. The DNA was subject to overnight restriction endonuclease digestion with endonucleases *EcoRI*, *NheI*, *NcoI*, *SphI* and *NsiI*, separated overnight by agarose gel electrophoresis and transferred to nylon membranes (Amersham). Probes were labelled with ³²[P]dCTP using the Megaprime DNA labelling system (Amersham) and hybridised to the membranes.

DNA methylation and X-inactivation

DNA from the patient and both her parents was cleaved with the methylation-sensitive restriction enzyme *HpaII* and the methylation status around the polymorphic trinucleotide repeats in the human androgen receptor gene was determined as described.¹³ X-inactivation study was performed on 5-bromodeoxyuridine (BrdU) (Sigma Aldrich) substituted chromosomes stained with the bis-benzimidazole dye Hoechst 33258 (Sigma Aldrich).¹⁴ The methylation pattern in the Prader-Willi region on chromosome 15 was determined by Southern blot analysis using *HpaII*-cleaved DNA from the patient and the PW71 probe.¹⁵

Expression analysis

RT-PCR analysis for different *ZDHHC15* transcript variants was performed on total lymphocyte RNA isolated from the patient and from control individuals using TRIzol solution (Invitrogen), and DNase treated using the DNA-free kit (Ambion). Analysis of tissue distribution for the different *ZDHHC15* transcript variants was performed on RNA from human placenta, liver, kidney, heart, lung and brain purchased from Clontech. RT-PCR analysis of the flanking genes *MGC23937* and *ABC7*, located centromeric of *ZDHHC15*, and *MAGEE2*, located telomeric of *ZDHHC15*, was performed in order to investigate a possible positional effect on the transcription of these genes. The sequences of all primers used are detailed in Table 1. Amplifications of β -Actin were performed on the same samples as internal control for the RT-PCR.

Mutation screening of *ZDHHC15* in XLMR families by sequencing

DNA from affected probands of 21 families segregating for nonsyndromic X-linked mental retardation with suggested linkage to Xq13, including MRX families 4, 13, 17, 22, 61, 65, 67 and 78, was collected within the frame-work of the European XLMR Consortium and Drs JL Mandel, Université Louis Pasteur, Illkirch, France, CE Schwartz, Greenwood Genetic Center, SC, USA, G Turner, University of Newcastle, Newcastle, New South Wales, Australia, MR Passos-Bueno, Instituto de Biociências – USP, Brazil and J Gecz, Women's and Children's Hospital, North Adelaide, Australia. PCR products of 300–400 nucleotides in size corresponding to the known coding sequences of the

Table 1 Primers used for RT-PCR amplification

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
<i>ZDHHC15.a</i>	TGCCTGGTACTGTTTTGAG	ATGCAGGCCACAAAGAGAAG
<i>ZDHHC15.b</i>	ATGCACACACTGGGTTTCAA	TGAATGCTTGGCAGAAGATG
<i>ZDHHC15.d</i>	GCTGGAAGATGGCTCTGTCT	TGGGTCTCTAAATGGCAGAAA
<i>ZDHHC15.e</i>	CTTCAAGAATGACATGGCAAC	GATAGACTTCCAGTAGGTCCAGGT
<i>MGC23937</i>	AGACCATCATCCGGGACA	GCCTCACCGCTTCTCATTAT
<i>ABCB7</i>	CGGGAAACATGCTGAACC	CCCAGATCCAGGTTGTGAAG
<i>MAGEE2</i>	CCACCTCCATGCTAGTCGT	CCAAGCAATCGACCTGTCT
β -Actin	CTGGAACGGTGAAGGTGACA	CGGCCACATTGTGAACCTTG

different human *ZDHHC15* transcripts were generated from all 21 DNA samples. Sequencing was performed using BigDye Terminator Chemistry (PE Biosystems) and an ABI 3700 DNA Analyzer (Applied Biosystems).

Results

Mapping of the translocation breakpoints

In order to localise the t(X;15) translocation breakpoints, we performed a series of FISH studies with genomic clones (Figure 1a). Two clones from the Xq13.3 region (RP11-451L9 and RP11-262B13) hybridised both to the normal chromosome X and to the two derivative chromosomes der(X) and der(15) (Figure 1b). These two clones overlap by 153 648 bp. A mini-FISH experiment with the *ZDHHC15* probe containing the first exon of the gene *ZDHHC15* restricted the breakpoint region to within 61 kb (Figure 1c and d). The chromosome 15 centromere-specific probe D15Z1 hybridised to the normal chromosome 15 and the der(X) chromosome, whereas the α -satellite probe hybridised, in addition to all normal chromosomes, to der(15) and at two separate loci on the der(X). This positions the chromosome 15 breakpoint within the α -satellite region of the centromere (results not shown).

Aberrant restriction fragments in patient DNA were observed in restriction digests with the endonucleases *NcoI*, *EcoRI* and *NheI* and using the probe Xso12 corresponding to nucleotides 95 182–95 469 of the clone RP11-451L9, located 1509–1796 bp upstream of the first exon of the *ZDHHC15* gene (Figure 2a and b). No aberrant fragments were observed with the *SphI* and *NsiI* restriction digests using the same probe. These results enabled us to locate the breakpoint in Xq13.3 to a 1643 bp *NsiI*–*NcoI* restriction fragment. This fragment is located 2242–3885 bp upstream of the first exon of the gene *ZDHHC15* (Figure 2c).

The *ZDHHC15* gene

According to the NCBI Aceview database (www.ncbi.nlm.nih.gov/AceView), the *ZDHHC15* gene is predicted based on alignment of mRNA and ESTs to genomic sequences. The coding sequences are supported by 32 sequences from

31 cDNA clones. Four identified nonoverlapping alternative last exons produce five different transcripts as a result of alternative splicing (Figure 3a). We were able to amplify the alternative last exon for all transcript variants except for variant *c* (not outlined in Figure 3a) as suggested by the NCBI Aceview database. In addition, the primers used to amplify the transcript variant referred to as '*ZDHHC15.a*' by the NCBI Aceview database identified four splice variants; '*ZDHHC15.a1*' with all annotated exons, '*ZDHHC15.a2*' lacking exon 4, '*ZDHHC15.a3*' lacking exon 7 and '*ZDHHC15.a4*' lacking both exons 4 and 7. Since exons 4 and 7 both consist of 121 nucleotides, the two corresponding spliced variants *a2* and *a3* were only detected by sequencing (Figure 3a).

Three of the transcript variants (*a1*, *a3* and *b*) contain the DHHC zinc-finger domain as predicted by the NCBI Conserved Domain database (<http://www.ncbi.nih.gov/Structure/cdd/cdd.shtml>). The PSORT II (<http://psort.nibb.ac.jp/form2.html>) and the TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) prediction servers suggest *ZDHHC15.a1* to have four transmembrane motifs at positions 19–35, 55–71, 173–189 and 213–229 and that the protein is localised in the endoplasmic reticulum (ER) (Figure 3b). The protein displays homology to those from other species including *Zdhhc15* (*Mus musculus*) (Figure 3c).

DNA methylation and X-inactivation

Analysis of DNA methylation at the androgen-receptor locus in Xq11–12 showed 100% skewed X-inactivation in samples from the patient indicating that the normal X chromosome is retained inactive. The X-inactivation pattern by late replication studies was also determined from an EBV-transformed lymphoblastoid cell line. BrdU incorporation in 68 studied metaphases showed that the normal X chromosome was late replicating in 81% of cells examined. In the remaining 19%, the X-inactivation status could not be determined. The methylation pattern in the Prader–Willi/Angelman region on chromosome 15 was investigated by Southern analysis and hybridisation with the probe PW71 showed a normal hybridisation pattern.

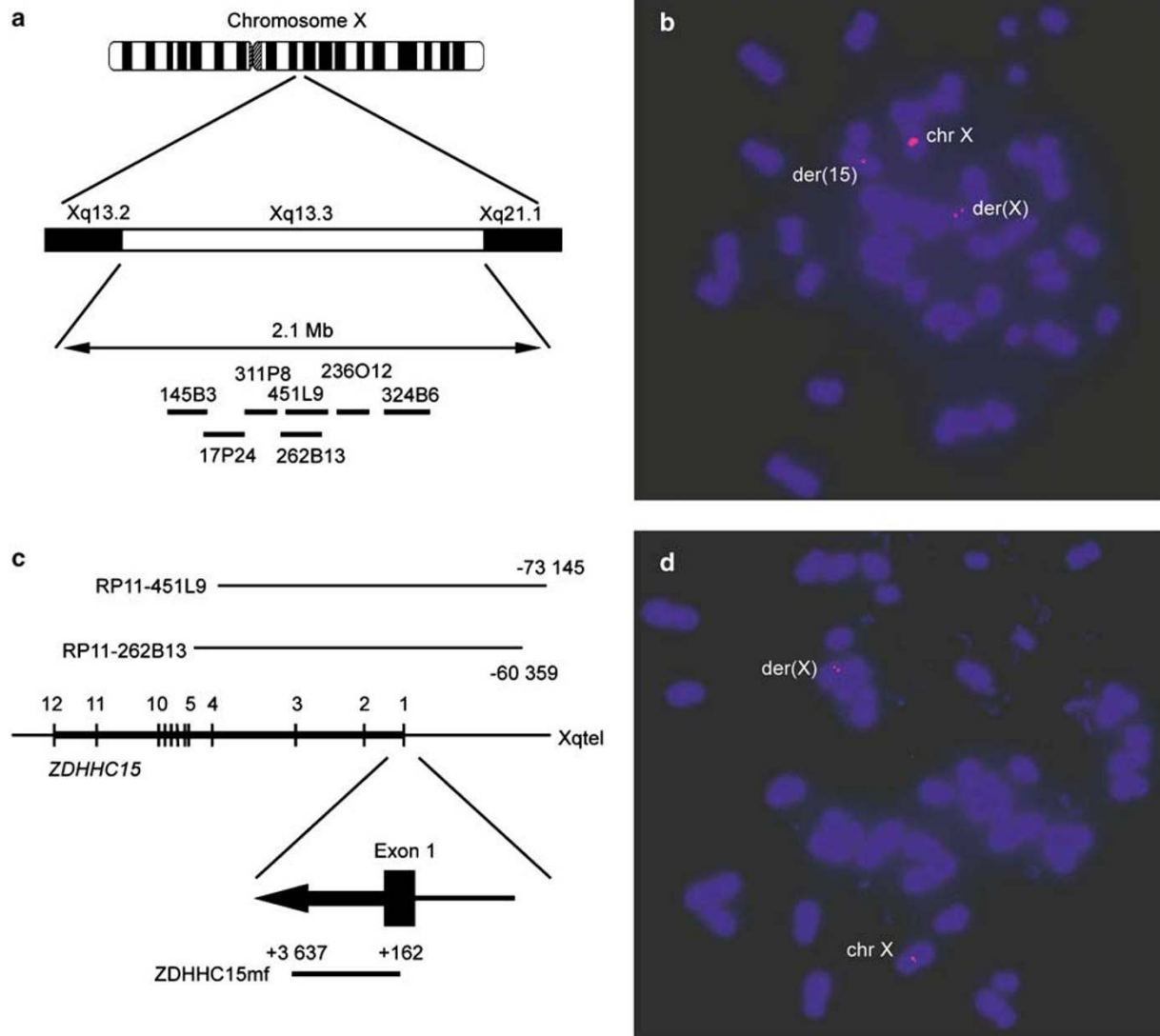


Figure 1 Mapping of the Xq13.3 breakpoint region involved in the t(X;15) translocation. (a) Physical map of the Xq13.3 region with corresponding genomic BAC clones used for FISH. (b) FISH with the clone RP11-451L9. Hybridisation signals can be detected on the normal chromosome X (chr X) and the two derivate chromosomes der(15) and der(X). (c) Schematic structure of the *ZDHHC15* gene with the corresponding BAC clones (RP11-451L9 and RP11-262B13) and the probe ZDHHC15mf used for mini-FISH. The numbers indicate the position of the DNA sequence relative to the first base of the *ZDHHC15* gene. (d) Mini-FISH with ZDHHC15mf. Signals are detected on the normal chromosome X and on the derivate X chromosome.

Expression studies and mutational screening

Transcript variant-specific RT-PCR did not reveal products from any of the tested *ZDHHC15* variants in primary lymphocytes from the patient. However, the tested variants *a1-4*, *b*, *d* and *e* were expressed in control samples (Figure 4a). The β -Actin derived RT-PCR products used as internal control were observed in all samples. The normal expression pattern of the different *ZDHHC15* transcript variants was assessed by tissue-specific RT-PCR using RNA derived from human placenta, liver, lung, kidney, heart and brain (Figure 4b). Expression pattern of the genes

MGC23937 and *ABCB7*, which are located centromeric of *ZDHHC15* and *MAGEE2* which is located telomeric of *ZDHHC15*, was similar in lymphocytes from the patient compared to normal controls (Figure 4c and d). The two PCR products amplified with the *MGC23937* primers are the result of alternative splicing of *MGC23937* with the larger fragment containing additional coding sequence between the second and the third exon.

Sequencing of DNA samples from affected members of families with nonsyndromic XLMR and suggested linkage to Xq13 identified five novel nucleotide exchanges and

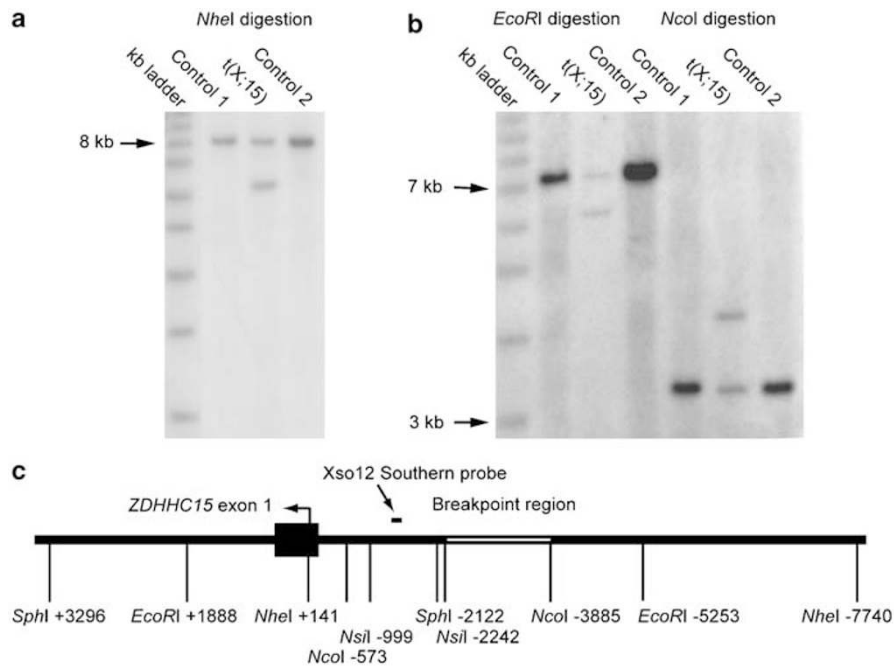


Figure 2 Mapping of the chromosome X breakpoint with Southern blot analysis. (a) Southern blot analysis with Southern probe Xso12 and *NheI* restriction digestion. (b) Southern blot analysis with Southern probe Xso12 and *EcoRI* and *NcoI*, respectively. (c) Physical map of the breakpoint region with *SphI*, *NcoI*, *NheI*, *EcoRI* and *NsiI* restriction sites.

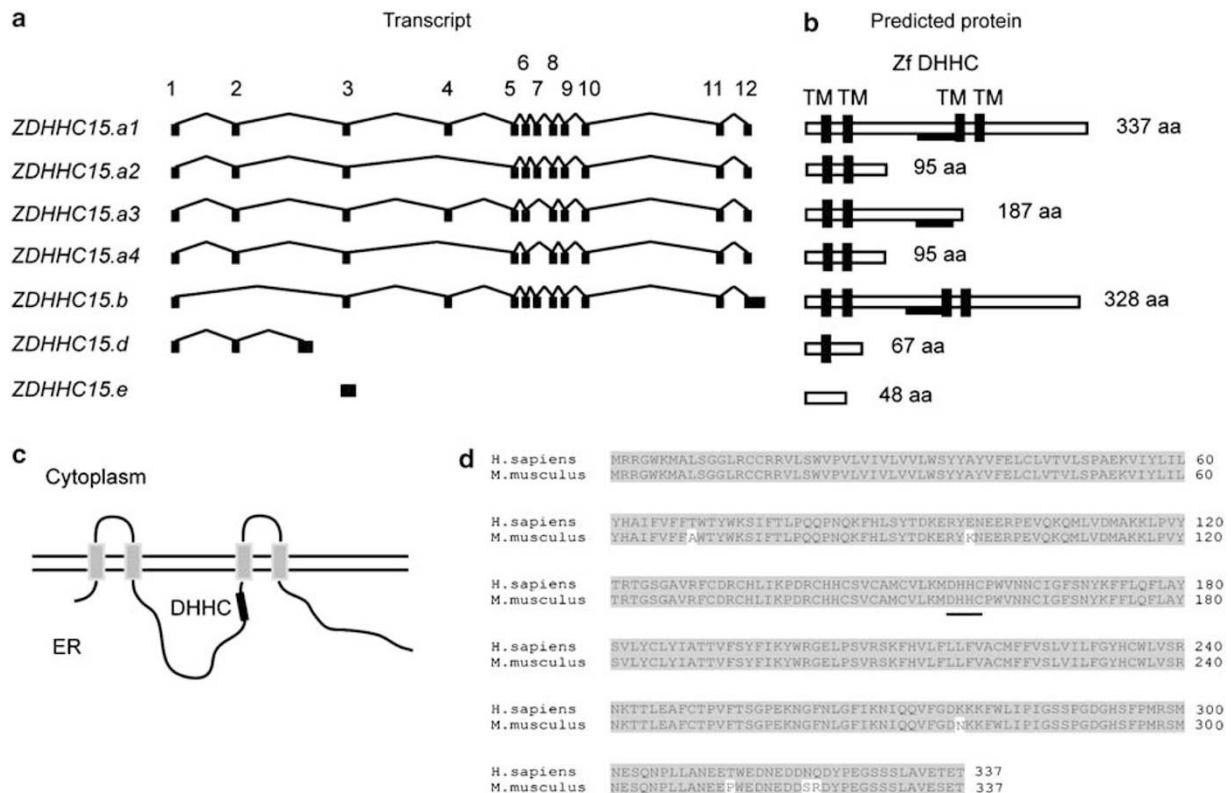


Figure 3 Structure of *ZDHHC15*. (a) Genomic structure and the predicted protein structure of all *ZDHHC15* transcript variants. The transmembrane (TM) and the DHHC domains are outlined. (b) Predicted structure of the *ZDHHC15.a1* protein. ER, endoplasmic reticulum. (c) Sequence alignment of the *ZDHHC15.a* protein with mouse *Zdhhc15* shows a high degree of conservation between the two species. The DHHC domain is underlined.

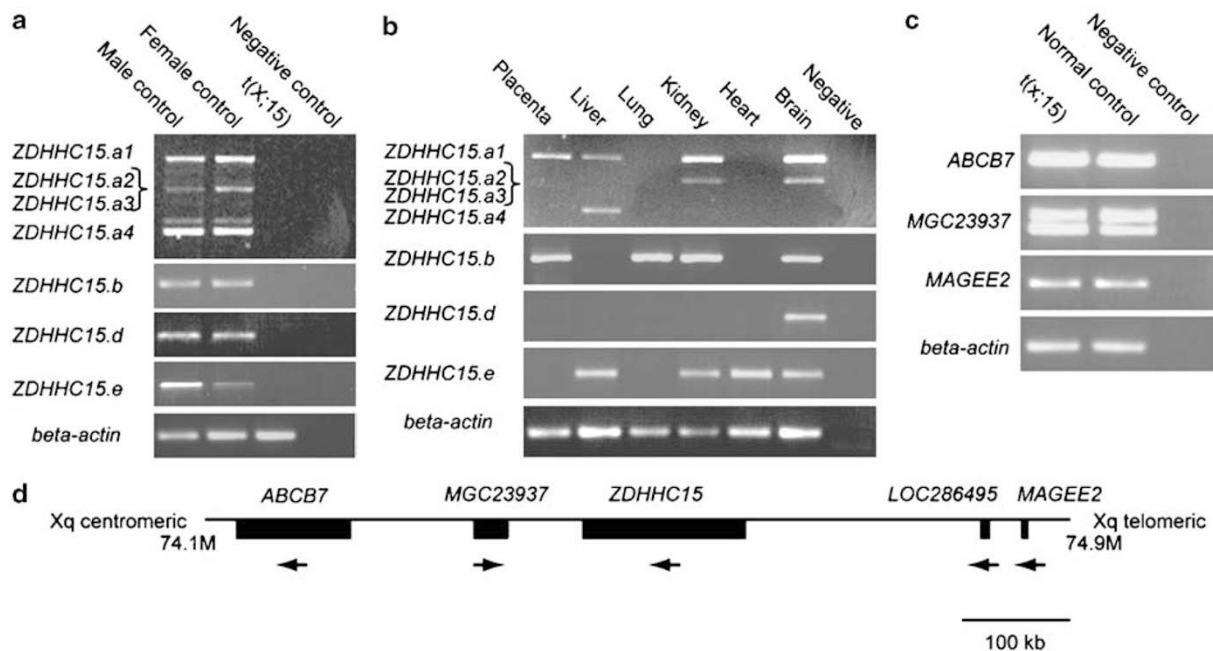


Figure 4 Expression analysis of *ZDHHC15* transcript variants from total RNA by RT-PCR. (a) Expression pattern of all *ZDHHC15* transcript variants in lymphocytes from control individuals and the patient. The β -Actin transcript is used as an internal control. The *ZDHHC15.a4* transcript is represented by two bands verified by sequencing. (b) Tissue distribution of the different *ZDHHC15* transcript variants illustrated by RT-PCR analysis. (c) RT-PCR analysis of the genes *ABCB7* and *MGC23937*, which are located centromeric of *ZDHHC15* and *MAGEE2* which is located telomeric of *ZDHHC15*. (d) Physical map of Xq13. The transcribed genes in the region are outlined.

Table 2 Sequence alterations identified in the 3' UTR of *ZDHHC15* in DNA from XLMR patients

Variant	Genbank accession no.	Exon	Sequence alteration ^a	No. of patients	No. of control chromosomes
<i>ZDHHC15.b</i>	AK122885	12	C2962T	1	0/100
<i>ZDHHC15.b</i>	AK122885	12	C3378G	1	3/94
<i>ZDHHC15.b</i>	AK122885	12	G3430T	1	3/94
<i>ZDHHC15.d</i>	AL580822	3	T576C	1	0/100
<i>ZDHHC15.d</i>	AL580822	3	Del724–727	1	4/100
<i>ZDHHC15.d</i>	AL580822	3	Del727–730	4	17/100
<i>ZDHHC15.e</i>	AL134778	1	C278T	1	1/94

^acDNA position.

two different 4bp deletions in the 3' UTR region of the various *ZDHHC15* transcript variants previously not reported. No alterations in protein coding sequences were detected (Table 2).

Discussion

In this study, we present the mapping of both chromosomal breakpoints from a balanced reciprocal translocation 46,XX,t(X;15)(q13.3;cen) associated with severe MR. The patient showed 100% skewed X-inactivation at the androgen receptor locus and the normal X chromosome was late replicating in at least 81% of cells examined, suggesting

that the structurally normal X is inactivated in the patient. The undetermined status of the remaining 19% cells by late replication analysis can be explained by the lack of synchronisation of the cell culture resulting in some cells having incorporated BrdU at slightly different time points during replication.

The X chromosome breakpoint was mapped to within 3.9 kb of the first exon of the *ZDHHC15* gene. RT-PCR analysis of the *ZDHHC15* gene in patient-derived lymphocytes indicates that transcription is turned off affecting all alternative transcript variants of *ZDHHC15*. These transcripts were clearly detected in control samples. The silenced transcription of *ZDHHC15* may be due to the separation of the promoter and transcription units from a

distant cis-acting regulatory element because of the translocation. The chromosomal rearrangement may also give rise to position effect variegation (PEV) which can occur when a chromosomal rearrangement causes the juxtaposition of a euchromatic gene with a region of heterochromatin.¹⁶ In such a case, the heterochromatin state of the DNA is believed to spread via formation of multiprotein complexes to the juxtaposed euchromatin region subsequently silencing the nearby gene.^{17,18} A PEV mechanism for the translocation was excluded for the region immediately flanking *ZDHHC15* as indicated by the normal expression of the flanking genes *MGC23937*, *ABCB7* and *MAGEE2*.

Interestingly, the patient displayed several features in common with Prader–Willi Syndrome (PWS) during infancy including obesity and muscular hypotonia and she was originally described as PWS-like.¹⁰ However, these features have disappeared with age and today, at 29 years of age, she does not present with PWS symptoms. In addition, the breakpoint on chromosome 15 is located at least 4.5 Mb centromeric of the PW region and a normal hybridisation pattern was found using the probe PW71. Taken together, the clinical and molecular investigations make the involvement of the PW region unlikely.

Sequencing analysis of 21 probands with nonsyndromic XLMR and suggested linkage to Xq13 revealed no mutation in the protein coding sequences of *ZDHHC15*.

Five of the nucleotide variations identified in the 3' UTR region of the different transcript variants were confirmed as polymorphisms whereas two were not found in 100 control chromosomes. These latter variations are neither excluded nor confirmed as polymorphisms. Mutations in genes associated with nonsyndromic XLMR may be found at a very low frequency. A mutation in *ARHGFB6* [MIM 300267], which is reported in nonsyndromic XLMR, was identified in one of 119 affected families screened.¹⁹ Shoichet *et al.*⁸ reported alterations in *ZNF41* [MIM 314995] in two out of 210 unrelated mentally retarded patients analysed. Therefore, more patients need to be investigated in search for XLMR-associated mutations in *ZDHHC15*.

Alternatively, an alteration of this gene in males may have a severe phenotypic effect possibly associated with male lethality. The phenotype in our patient may result from a leaky X-inactivation pattern and the observations made would possibly represent XLMR confined to female subjects or possibly her alone.

Yeast ZDHHC proteins AKR1 and ERF2 are transmembrane palmitoyl transferases.^{20,21} Recently, the huntingtin-interacting protein ZDHHC17, which is a member of the ZDHHC protein family, was shown to be a neuronal palmitoyl transferase suggesting that ZDHHC15 could function as a palmitoyl transferase with a yet unidentified substrate.²² Palmitoylation is the process of post-translational modification of proteins with the lipid palmitate.

Several classes of neuronal proteins, including proteins important for neuronal development, neurotransmitter receptors, secreted signalling molecules and synaptic scaffolding proteins are reversibly modified by palmitate.²³

From our combined results, we suggest that *ZDHHC15* is a strong candidate for nonsyndromic XLMR. The high degree of conservation of this gene between different species implies an important role in development. Further studies, including the screening of a larger group of patients with MR, are needed to clarify an association with *ZDHHC15* and its potential role in cognitive function.

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