A novel 2 bp deletion in the *TM4SF2* gene is associated with MRX58

F E Abidi, E Holinski-Feder, O Rittinger, F Kooy, H A Lubs, R E Stevenson, C E Schwartz

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Xlinked mental retardation (XLMR) represents around 5% of all MR, with a prevalence of 1 in 600 males.^{1,2} Fifteen to twenty percent of the total XLMR is the result of the fragile X syndrome.³ Non-fragile X mental retardation was subdivided into syndromal and non-syndromal conditions by Neri *et al*⁴ in 1991. The syndromal XLMR entities (MRXS) are those in which there is a specific pattern of physical, neurological, or metabolic abnormalities associated with the presence of mental retardation.⁵ Non-syndromic XLMR (MRX) are conditions in which a gene mutation causes mental retardation in the absence of other distinctive dysmorphic, metabolic, or neurological features.⁶ At present, XLMR conditions consist of 136 MRXS⁷ and 75 MRX⁸ entities. To date, 35 genes have been cloned. However, so far only nine non-syndromic XLMR genes have been identified: *TM4SF2*, *FMR2*, *OPHN1* (MRX60), *GDII* (MRX41, MRX48), *PAK3* (MRX30, MRX47), *RSK2* (MRX19), *IL1RAPL* (MRX34), *ARHGEF6* (MRX46), and *MECP2* (MRX16).^{9–18}

TM4SF2, a member of the transmembrane 4 superfamily, maps to Xp11.4 and is one of the genes associated with non-syndromic XLMR.⁹ Mutations in *TM4SF2* have been previously described in two families (L28 and T15) with non-syndromic XLMR. As a part of our XLMR candidate gene testing, we have screened probands from 14 XLMR families (10 linked to Xp11 and four small families with no linkage data) for mutations in the *TM4SF2* gene. Here we report a novel 2 bp deletion (564delGT), which segregates with mental retardation in the MRX58 family. The deletion causes a frameshift and a subsequent stop codon six amino acids downstream (stop codon 192). This finding supports the hypothesis that different XLMR conditions, especially non-syndromic XLMR, result from mutations in the same gene.

MATERIAL AND METHODS

Patients

Probands from 14 XLMR families (three MRXS and 11 MRX) were used in this study. The three linked MRXS families consisted of MRXS10¹⁹ and two unpublished families. Of the seven linked MRX families, five families (MRX12, MRX18, MRX33, MRX56, MRX58) have been published previously,^{20–24} while two families are unpublished. The remaining four XLMR families were small and no linkage analysis had been done.

Incorporation PCR SSCP (IPS)

Genomic DNA was isolated from lymphocytes using a high salt procedure.²⁵ IPS was performed using 50 ng of genomic DNA in a total volume of 10 µl containing 1 × PCR reaction buffer (10 mmol/l Tris, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, pH 8.3) with 1 µmol/l of each primer, 50 µmol/l of dNTPs, 0.05 µCi of α³²P dCTP (3000 Ci/mmol, NENTM Life Science), and 0.5 units of *Taq* DNA polymerase (Sigma).²⁶ The information on the PCR primers and their optimal annealing conditions are listed in table 1. The primers were designed to be approximately 50 bp away from the exon/intron boundary to ensure

detection of any alteration at the splice junction. Since the PCR products for exons 2 and 5 were greater than 310 bp, they were digested with *Hae*III and *Pvu*II respectively (table 1). Amplification was done in a PTC-200 thermocycler (MJ Research). Following PCR, 10 µl of the IPS loading dye (95% formamide, 10 mmol/l NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) was added. The samples were denatured at 96°C for three minutes and loaded on a 0.5 × MDE gel (FMC) prepared in 0.6 × TBE. The gel was run at 8 W for 15–20 hours at room temperature. The gel was dried and the radioactive signal was visualised using the BIOMAXTM MS films (Eastman Kodak).

Sequence analysis

PCR products that showed an abnormally migrating pattern on IPS were sequenced directly using the fmol kit (Promega) according to the manufacturer's protocol. Sequences were analysed using a 6% denaturing gel containing 6 mol/l urea. The gel was dried and the radioactive signal was visualised using the BIOMAXTM MS films (Eastman Kodak). The primers used for the PCR amplification were also used for sequencing.

Mutation analysis

The 564delGT in MRX58 created a *Bsr*I site in the altered sequence. The forward and reverse primers for exon 5 were used to amplify the region spanning the deletion (table 1). The 371 bp of amplified fragment was then digested with *Bsr*I (NEB) and the digestion products were run on an 8% acrylamide gel.²⁷ Upon digestion, the normal sequence yielded fragments of 137 bp, 149 bp, and 85 bp, whereas the sequence carrying the deletion resulted in fragments of 137 bp, 95 bp, 52 bp, and 85 bp.

RESULTS

We have screened the seven exons of the *TM4SF2* gene, using IPS combined with sequencing of the PCR product in 14 XLMR families. Two populations of IPS shifts were observed in exon 5, with the patient from MRX58 having a different shift from the other samples (indicated by arrow 1 in fig 1A). Sequencing of exon 5 from the MRX58 patient showed that he was carrying a 2 bp (GT) deletion at position 564 (564delGT, fig 1B). This deletion results in a frameshift giving rise to a stop codon six amino acids downstream (stop codon 192). The deletion falls in the second extracellular domain and results in a truncated protein missing the fourth transmembrane as well as the carboxy end of the gene. The deletion created a *Bsr*I restriction enzyme site in the abnormal sequence, making it possible to test for cosegregation in the family. This analysis determined that the deletion segregated with the XLMR in MRX58 (fig 2).

Abbreviations: MR, mental retardation; XLMR, X linked mental retardation; MRXS, syndromal XLMR; MRX, non-syndromal XLMR; IPS, incorporation PCR SSCP

Figure 2 Polyacrylamide gel electrophoresis of *BstI* restriction enzyme digests of amplified DNA fragments from the members of the MRX58 family showing segregation of 564delGT. A partial pedigree of the MRX58 family is shown. The normal males have 149 bp, 137 bp, and 85 bp bands, whereas the affected males have 137 bp, 95 bp, 85 bp, and 52 bp bands.

uninformative in the two families tested (MRX33 and MRX56), since the carrier females were homozygous for this change (data not shown).

DISCUSSION

Here we report a 2 bp deletion (564delGT) in the *TM4SF2* gene, which segregates with XLMR in MRX58.²⁴ This family has five affected males in two generations with mild to moderate mental retardation. All affected males live in sheltered homes, are capable of doing simple jobs, and have no dysmorphic features. The carrier females in the family have normal intelligence. X inactivation studies indicated a random pattern (45:55) in all three.

The *TM4SF2* gene is inactivated by a balanced translocation (X;2) in a female patient with mild MR associated with minor autistic features.⁹ A missense (Pro172His) mutation and a nonsense (Gly218X) mutation in the *TM4SF2* gene have been previously reported in two small families, L28 and T15.⁹ Affected males from these two families have non-syndromic XLMR with no dysmorphic features, which was also observed in the MRX58 family. All three mutations are in the second extracellular domain of the *TM4SF2* gene. The 2 bp deletion found in MRX58 and the nonsense mutation reported in family T15 remove a similar portion of the *TM4SF2* gene.⁹ This finding provides further evidence that one gene could be responsible for several of the MRX conditions published.

The *TM4SF2* gene encodes a member of the tetraspanin family of proteins. These are cell-surface proteins that span the membrane four times and form two extracellular domains.⁹ The proteins have the ability to associate with one another and with $\beta 1$ -integrins and class I and class II HLA proteins.²⁸ Their interaction with $\beta 1$ -integrins might activate Rac and RhoGTPases and mediate several cellular processes, such as the regulation of actin cytoskeleton dynamics, the activation of signalling pathways, and cell proliferation, adhesion, and migration.²⁹ *TM4SF2* is highly expressed in the cerebral cortex and hippocampus including the primary olfactory cortex, though very little is known about the function in the physiology of CNS.⁹

The number of genes on the X chromosome responsible for non-syndromic XLMR was first estimated by Herbst and Miller as seven to 19 in 1980.² Their estimate was predicted on a prevalence estimate of 1.83 cases of non-syndromic XLMR/1000 males. Their estimate appeared to be reasonable based on the ability of later researchers to group the localisations of MRX conditions into either eight or 10-12 non-overlapping loci depending on the criteria used to determine limits of the published localisations.²⁰⁻³⁰ However, it is now evident that the number of genes responsible for the 75 presently known MRX conditions will probably exceed the upper limit of 19 proposed by Herbst and Miller.² This conclusion is based on results recently generated in various laboratories. For example, in Xq28, which contains at least nine MRX conditions (MRX3, MRX6, MRX16, MRX25, MRX28, MRX41, MRX46, MRX48, MRX72³¹⁻³²), two genes have been found that account for only three of these conditions, *GDI1* (MRX41, MRX48)¹² and *MECP2* (MRX16).¹⁸ This would mean at least one more gene must exist in Xq28. A similar situation exists for the Xq22-q23 region where *PAK3* is found to be mutated in MRX30 and MRX47, but not in another seven MRX entities whose localisations span this region. Thus, the minimum number of MRX genes is likely to be three times the number of non-overlapping loci defined by Lubs *et al.*³⁰

However, even this rationale for estimating the number of MRX genes is complicated by the finding that genes associated with syndromic XLMR conditions may also be involved in MRX. *RSK2*, the gene associated with Coffin-Lowry syndrome, is mutated in MRX19,¹⁵ *MECP2*, the Rett syndrome gene, is mutated in MRX16,¹⁸ and *FGD1*, the Aarskog gene, has been found to have a mutation in a family with non-syndromic XLMR.³³ Therefore, to determine the number of genes on the X chromosome responsible for MRX conditions it will be necessary to screen every known candidate XLMR gene in all of the known linked XLMR families, MRX and MRXS, within the localisation interval.

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Authors' affiliations

F E Abidi, R E Stevenson, C E Schwartz, J C Self Research Institute, Greenwood Genetic Center, Greenwood, SC, USA
E Holinski-Feder, Medizinisch Genetisches Zentrum, Bayerstrasse 53, D-80335, München, Germany
O Rittinger, Landeskrankenanstalten Salzburg, Kinderspital, Klinische Genetik, Salzburg, Austria
F Kooy, Department of Medical Genetics, University of Antwerp, Antwerp, Belgium
H A Lubs, Pediatrics/Genetic Division University of Miami, Miami, FL, USA

Correspondence to: Dr C Schwartz, Center for Molecular Studies, J C Self Research Institute, One Gregor Mendel Circle, Greenwood, SC 29646, USA; schwartz@ggc.org

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