SHORT REPORT

Abnormal expression of the *KLF8* (*ZNF741*) gene in a female patient with an X;autosome translocation t(X;21)(p11.2;q22.3) and non-syndromic mental retardation

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J Med Genet 2002;39:113–117

Non-syndromic X linked mental retardation (MRX) is a heterogeneous group of conditions in which all patients have mental retardation as the only constant phenotypic feature. We have identified a female patient with mental retardation and a balanced translocation involving chromosomes X and 21, t(X;21)(p11.2;q22.3). Physical mapping of the translocation breakpoint on the human X chromosome was performed using fluorescence in situ hybridisation. We have mapped the X chromosome breakpoint to a 21 kb DNA fragment upstream of the first exon of the *KLF8* (*ZNF741*) gene in Xp11.21. We have subsequently shown that the *KLF8* transcript is no longer detected in cells from the patient, although *KLF8* expression is otherwise normally present in control lymphoblasts. Mutation screening of probands from 20 unrelated XLMR families linked to the proximal short arm of the human X chromosome failed to show any mutation in the coding region of the *KLF8* gene.

Non-syndromic X linked mental retardation (MRX) is a very heterogeneous group of conditions in which all patients have mental retardation as the only distinguishing phenotypic feature. During the past few years, several MRX genes have been identified: *FMR2*,^{1,2} *GDII*,³ *OPHN1*,⁴ *PAK3*,⁵ *RPS6KA3*,⁶ *IL1RAPL1*,⁷ *TM4SF2*,⁸ and *ARHGEF6*.⁹ Despite the large number of familial cases of non-syndromic mental retardation available for study, systematic mutation screening in these genes has failed to find a major locus for this group of conditions. Only a few mutations have been described in each of the MRX genes (six mutations identified among the 76 MRX families currently recognised by the HUGO gene nomenclature committee, <http://www.gene.ucl.ac.uk/nomenclature/>). The number of genes that could be responsible for a MRX phenotype based on overlapping linkage intervals has previously been discussed.¹⁰ However, the currently available molecular data seem to show that no major locus exists; rather, probably, a large number of X linked genes can cause a non-syndromic mental retardation phenotype when mutated.

Three MRX genes (*OPHN1*, *TM4SF2*, *ARHGEF6*) were identified on the basis of their interruption by X;autosome translocations in female patients with mental retardation. The normal X chromosome is usually inactivated in the cells of females with such a balanced chromosomal rearrangement to maintain a correct dosage in the autosomal regions translocated to the X chromosome.¹¹ Hence, any gene interrupted by the X chromosome breakpoint is potentially responsible for the phenotype observed in the patient.

We have identified a female patient with mental retardation and a de novo balanced translocation involving chromosomes X and 21. Karyotype analysis showed a t(X;21)(p11.2;q22.3). We focused our attention on the X chromosome in order to determine if a gene was interrupted in the patient's DNA. This choice was dictated by the fact that a significant number of X linked mental retardation (XLMR) families are genetically linked to the proximal short arm of the human X chromosome and that any X linked gene that was interrupted in the patient would be a good XLMR candidate gene.

We have mapped the X chromosome breakpoint region to a 21 kb restriction fragment which is located immediately upstream of the first exon of the *KLF8* gene. *KLF8*¹² is a CACCC box binding protein that associates with the transcriptional corepressor C-terminal binding protein (*CtBP*)¹³ and represses transcription. It contains three kruppel-like zinc finger motifs at its C-terminus. The *KLF8* gene is expressed ubiquitously.

We determined that the *KLF8* transcript is absent from lymphoblasts of the translocation patient although it is easily detected in normal controls. This fact prompted us to search for *KLF8* mutations in 20 XLMR families linked to this region of the human X chromosome. Unfortunately, no disease causing mutation was identified in this panel of families. Therefore, it is unlikely that *KLF8* is responsible for a large proportion of X linked mental retardation.

MATERIAL AND METHODS

Case report

The patient is the fourth child of healthy, unrelated parents. She was referred at the age of 5 years because her psychomotor development was delayed. Birth weight was 3400 g, length was 48 cm, and OFC was 35 cm. Apgar scores were 10 at one and five minutes. At the age of 9 months, she presented with seizures which never recurred. She walked at the age of 2 years and began speaking in short sentences at 4 years. Aged 5 years, she cannot run nor stand on one leg. She cannot stand up alone from a sitting position. Examination at this age showed genu valgum, pelvic muscle atrophy, and global hypotonia. Her development was evaluated to be 2.5 years for motor skills and adaptation and 4 years for language and sociability. At 5 years, her weight was at +4 SD and her height at +1.5 SD. Blood examination, routine biochemical analysis, nerve conduction velocity, and MRI were normal. Karyotype analysis showed a de novo balanced translocation t(X;21)(p11.2;q22.3).

Abbreviations: MRX, non-syndromic X linked mental retardation; XLMR, X linked mental retardation

Table 1 Oligonucleotides used to amplify the six exons of *KLF8* using genomic DNA as a template

Exon	Forward (5'-3')	Reverse (5'-3')	Exon size	Intron size
1	GCCGGAAGTGAATTGGTAGG	AGGGGGATAGAATCTAGACG	436 bp	17.8 kb
2	GAACACACATAACATTAATGGC	AAATTCTGTAATCACTACCAGG	74 bp	15.9 kb
3	TGCTTGCATGTCTCTTCAGG	GATAGGATGACTCTAAAAGGG	565 bp	4.8 kb
4	TTAAGGTGGGACAACTGGG	GTTCCACCTATACATATGTGC	112 bp	0.7 kb
5	GCCGATATGATGTGGCACC	TCCATGAATGGAGCCTGTGC	140 bp	10 kb
6	CTCTAGTATCCGATCATCC	GCAAGCTGAAGTCAAAGAGG	872 bp	–

XLMR families

Ten probands came from the Greenwood-Miami collection of XLMR families. These consisted of one male each with *MRX7*,¹⁴ *MRX8*,¹⁶ *MRX17* Miles-Carpenter syndrome,¹⁸ and Stocco dos Santos syndrome,²⁰ and families K8360, K8820, K8825, K8835, K8900, and K8923 (C Schwartz, unpublished data). K8360 is a MRX family localised between *MAOA* and DXS990, K8820 a MRX family localised between DXS8015 and DXS995, K8825 a small XLMR family in which males have relative macrocephaly and prominent ears, and a broad localisation between DXS999 and DXS424, K8835 is a MRX family with localisation between *RBM3* and DXS990, K8900 is a XLMR family in which the affected males have growth impairment, marked developmental delay (no speech or ambulation), seizures, athetosis, and spasticity, and a broad localisation between DXS993 and DXS1002, and K8923, previously reported²² as having features of Renpenning syndrome,²³ has been localised between DXS8035 and DXS424. The eleventh case is a patient from the *MRX9* family localised between DXS164 and DXS453.²⁵ Nine additional families came from Australia. These include five previously published MRX families, *MRX1*, *MRX12*, *MRX13*, *MRX17*, and *MRX31*,¹⁰ one unpublished family (A Gedeon, unpublished data), and three families with syndromic features²⁶ (A Gedeon, unpublished data). X chromosome map localisation for all these families overlaps that of the position of the *KLF8* gene.

X chromosome inactivation analysis

Replication studies of the X chromosomes of cells from the patient were performed using a very late terminal BrdU pulse for the last three hours of culture. Chromosomal DNA was denatured in 70% formamide for two minutes at 70°C, then covered with a fluorescein conjugated anti-BrdU antibody to a final concentration of 25 µg/ml in PBS, and incubated for 60 minutes at room temperature. The slides were then washed 3 × 5 minutes in PBS, and the chromosomes were counterstained with DAPI. Hybridisation with an X alloid probe was performed simultaneously. Molecular analysis of X chromosome inactivation at the *AR* locus was performed as described elsewhere.²⁷

Fluorescence in situ hybridisation (FISH)

The probes (DNA from YACs, PACs, and restriction fragments) were labelled by random priming with bio-16-dUTP and in situ hybridised at a final concentration of 20 ng/µl. The hybridisation signals were made visible with fluorescein labelled avidin following standard protocols.²⁸ Chromosomes were counterstained with propidium iodide diluted in pH 11 antifade.

In silico analysis

The Nix genomic sequence analysis program at the UK Human Genome Mapping Project (<http://www.hgmp.mrc.ac.uk/NIX/>) was used for in silico analysis. Sequence comparisons were performed using the Blast algorithm at NCBI (<http://www.ncbi.nlm.nih.gov/blast/>).

Figure 1 Fluorescence in situ hybridisation of metaphase chromosomes from the translocation patient using PAC dJ148M19 as a probe. The PAC clone hybridises with the normal X chromosome and the two translocated chromosomes, a result expected for a clone spanning the translocation breakpoint.

RNA preparation and RT-PCR experiments

PolyA+ RNA was prepared using lymphocytes and the Quick-Prep mRNA purification kit according to the instructions of the manufacturer (Pharmacia). Reverse transcription of 500 ng of mRNA was performed in 50 µl of 1 × Superscript reaction buffer (Gibco BRL) containing 3 ng/µl of dN6, 40 units of Rnasein (Promega), 1 mmol/l dNTP, and 200 units of Superscript II reverse transcriptase (Gibco BRL). RT-PCR was performed using 1/10th of the first strand reaction.

Mutation screening in XLMR probands

The *KLF8* gene was sequenced for each patient using genomic DNA as a template. Primers were designed to amplify each exon of the gene (table 1). The PCR samples were directly sequenced, without subcloning, after purification with a Qia-gen PCR purification kit, using a LiCor DNA sequencer and M13 tailed primers.

RESULTS

X chromosome inactivation analysis

Metaphase spreads of lymphocytes of the patient with the X;21 translocation were studied for X chromosome inactivation. Forty cells were examined on three different slides (120 cells in total) and in all cases the normal X chromosome was found to be inactivated. This result was confirmed with a PCR assay using the polymorphic repeat sequence present in the androgen receptor gene, which showed a totally skewed X chromosome inactivation pattern (data not shown).

Mapping of the X chromosome Xp11.21 breakpoint using FISH

In order to map the translocation breakpoint on the X chromosome, we initially used the karyotype information and

Figure 2 Schematic representation of the translocation breakpoint region in Xp11.21. (Above) Physical map of the Xp11.21 region²⁹ (not to scale) with the YAC clones which were used for FISH experiments in our study. PAC clone dj148M19 is also shown. (Below) Structure of the dj148M19 PAC clone showing the five *KLF8* exons with their sizes and the sizes of the introns (not to scale), the localisation of the 21 kb *KpnI* fragment (shaded area) which spans the breakpoint region, and the position of DXS741.

a set of four overlapping YAC clones selected in a contig spanning the Xp11.21 genomic region.²⁹ We initially selected four YAC clones for further analysis: 857_g_12, 662_c_2, 945_e_12 and 973_d_2. These YACs were used to perform FISH experiments on chromosomes of the translocation patient. Two of them (YACs 945_e_12 and 857_g_12) were found to cross the translocation breakpoint (data not shown). To narrow the breakpoint region further, we next used in the same type of experiment PAC clones originating from a bacterial clone contig constructed by the Sanger Center in the same region (<http://www.sanger.ac.uk/HGP/ChrX>). PAC dj148M19 was found to cross the translocation breakpoint (fig 1). This PAC clone, positive for marker DXS741, has a size of 177 kb and its sequence is available (Genbank accession number AL050309). We digested the dj148M19 clone with the *KpnI*, *HindIII*, and *EcoRI* restriction endonucleases and the different restriction fragments were excised from agarose gels and used once again in FISH experiments. A 21 kb *KpnI* restriction fragment was found to cross the translocation breakpoint (fig 2).

The *KLF8* transcript is absent from the translocation patient cells

In silico analysis of the PAC dj148M19 sequence was performed using the Nix genomic sequence analysis tool (see Methods). This analysis showed that the PAC contained exons 1 to 5 of the *KLF8* gene (fig 2). No other obvious transcription unit was detected using various gene feature prediction programs or by comparison with the transcribed sequences databases (data not shown). The 21 kb *KpnI* restriction fragment derived from PAC dj148M19, which crosses the breakpoint, is located immediately upstream of the first exon of the *KLF8* gene. We thus wanted to determine if *KLF8* was properly expressed in cells from the patient. For this purpose, we performed multiplex RT-PCR experiments using primers specific for the *KLF8* cDNA together with primers from a ubiquitously expressed gene located outside the breakpoint region (the *XNP/ATR-X* gene in Xq13.3). Our results show that the *KLF8* transcript is almost totally absent in the patient's lymphocytes although it is easily detected in a control sample (fig 3).

Figure 3 *KLF8* expression analysis using RT-PCR. Two cDNA fragments were simultaneously amplified using the father, mother, or patient lymphocyte mRNA as a starting material. PC: positive control (mRNA from an unrelated normal subject). NC: negative control (no mRNA added to the reaction). In each case, the genomic DNA cannot be amplified using these PCR conditions and the size of the amplified fragments correspond to the *KLF8* (180 bp) and *XNP/ATR-X* (450 bp) cDNAs. Although the translocation patient sample was overloaded, this experiment shows that the *KLF8* transcript is almost totally absent in the patient's lymphocytes (a very faint band is visible).

KLF8 is composed of six exons and exon 2 is alternatively spliced

In order to explain why the *KLF8* transcript could not be detected in cells from the translocation patient, we tested for the potential presence of unknown additional 5' exons. For this purpose, we first checked that the published sequence of *KLF8* was correct. RT-PCR amplification of the *KLF8* transcript together with database (ESTs and genomic sequence) comparisons showed that nucleotides 1-254 of the *KLF8* cDNA

(GenBank entry NM_007250) do not belong to the *KLF8* transcript but rather correspond to a sequence on chromosome 2 (data not shown). The *KLF8* cDNA reported in GenBank is thus a coligated clone. Analysis of the *KLF8* ESTs (Unigene cluster Hs.320861) showed no evidence for any additional 5' sequence. We performed RT-PCR experiments in the 5' region of the *KLF8* transcript using human fetal and adult brain cDNA libraries, a *KLF8* specific primer, and primers corresponding to the cDNA library cloning vector. We were unable to extend the 5' sequence but these experiments showed that exon 2 of *KLF8* is alternatively spliced (data not shown). In addition, sequence comparison between the *KLF8* cDNA sequence, PAC clone dJ148M19, and an overlapping BAC clone (bB188A5) allowed us to show that *KLF8* is composed of six exons spanning 50 kb of genomic DNA in Xp11.21.

Mutation screening in MRX families

The fact that the *KLF8* gene was not found to be expressed in cells from the translocation patient using RT-PCR made it a good candidate for XLMR. In order to test this hypothesis, we screened a cohort of 20 unrelated XLMR families linked to the same interval of the human X chromosome for mutations in the gene (see Methods). Direct sequencing of each exon of the *KLF8* gene (table 1) using genomic DNA as a template was performed. This analysis failed to show any disease causing mutations.

DISCUSSION

We have precisely mapped the X chromosome breakpoint of a female patient with an X;autosome translocation t(X;21)(p11.2;q22.3) and mental retardation. The breakpoint is contained within a 21 kb *KpnI* restriction fragment originating from PAC clone dJ148M19 whose complete sequence was available. Two observations lead us to think that we may have found the molecular basis of the mental retardation of the patient: (1) numerous XLMR families are linked to this region of the human X chromosome and (2) PAC dJ148M19 contains the *KLF8* gene as its single transcription unit. Additionally, RT-PCR experiments determined that no transcripts of the *KLF8* gene are present in lymphocytes from the patient although this transcript is easily detected in control lymphocytes. These results prompted us to look for *KLF8* mutations in the DNA of 20 unrelated XLMR probands originating from families linked to the proximal short arm of the human X chromosome. However, we did not find disease causing mutations in these subjects after sequencing each exon of the *KLF8* gene.

There are some important implications from these results. If the *KLF8* gene is indeed responsible for the mental retardation observed in our patient, the lack of gene mutations in the 20 unrelated XLMR patients would mean that mutations in *KLF8* are not a frequent cause of XLMR. However, it should be pointed out that the other genes for which a causative effect on mental retardation has been proven are found mutated in only approximately 2% of the screened FRAXA negative males with MR. It is thus possible that we have not screened enough cases to identify a potentially disease causing mutation in another family. Many more cases should be screened in order to rule out this alternative hypothesis. In addition, we have not performed expression studies on the *KLF8* mRNA in the 20 families studied, since RNA samples were not available, and we thus cannot rule out the possibility that transcription is altered in one or several of these families.

On the other hand, since we are dealing with an X;autosome translocation, it is still possible that the patient's mental retardation is the result of a molecular defect (for example, an interrupted gene) on chromosome 21. If the involvement of the X chromosome is ruled out, this alternative hypothesis needs to be considered since the 21q22.3 region has been shown to be associated with some of the features of

Down syndrome, especially mental retardation.³⁰ To examine this possibility further, a detailed physical mapping of the chromosome 21 breakpoint will need to be carried out. Even if a gene is not found to be broken by the breakpoint on chromosome 21, there is the possibility that a nearby gene was put under the control of a different promoter. Since the breakpoint on the X chromosome maps in the promoter region of the *KLF8* gene, and since this gene is not expressed any more in the patient's cells, it may be that a gene on chromosome 21 has been placed under the control of the *KLF8* promoter or that the *KLF8* gene is now expressed under the control of a promoter associated with a gene on chromosome 21. Further studies will be needed to determine which hypothesis is true.

ACKNOWLEDGEMENTS

We would like to thank Drs Rogers, Thibodeau, Michels, Carpenter, Birnbaum, Bird, Hoganson, Toriello, Stocco dos Santos, O Phillips, J Ward, L Wolff, and C Munn for contributing families. Dr Abidi, D Tackels-Horne, R Nelson, M Chandler, and L Holmes assisted with the linkage analysis. We also thank Birgitta Winneppenninckx for helpful comments. This work was supported in part by the Inserm PROGRES network.

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Revised version received 25 October 2001

Accepted for publication 1 November 2001

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